ORIGINAL SUBMISSION

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nutrition&health

Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

Re: GRAS notification for Tonalin® Conjugated Linoleic Acid (CLA)

June 3, 2004

To Whom It May Concern:

Pursuant to proposed 21 CFR 170.36(c)(1)(62 Federal Reg. 189398, 18961; April 17, 1997) Cognis Corporation hereby provides notice of a claim that the food ingredient Tonalin® CLA is exempt from the premarket approval requirement of the Federal Food, Drug, ad Cosmetic Act because it has been determined to be Generally Recognized As Safe (GRAS), based on scientific procedures, for addition to food as an ingredient to provide consumers with a supplementary source of CLA in their diets.

GRAS Exemption Claim

Cognis Corporation hereby claims that Tonalin® Conjugated Linoleic Acid (CLA), for addition to specified foods (as described more fully in item (iii) below), is exempt from the premarket approval requirement of the Federal Food, Drug, and Cosmetic Act because Cognis has determined that Tonalin® CLA is Generally Recognized As Safe (GRAS) for such use.

(i) Name and address of notifier: Cognis Coroporation
5325 South Ninth Avenue
La Grange, IL 60525

(ii) ***Common or Usual Name of the Substance:*** Conjugated linoleic acid.

(iii) ***Applicable Conditions of Use:*** Tonalin® CLA is intended for use as an ingredient to provide consumers with a supplementary source of CLA in their diets in specified foods as summarized in the following table.

Food Group	mg CLA per serving
Yogurt	1680
Meal Replacement Beverages	1680
Meal Replacement Bars	1680
Fruit Juices	1680
Milk-based fruit drinks	1680
Milk-based beverages	1680
Liquid Cream Substitute	400
Powdered Cream Substitute	400
Milk Chocolate	400

(iv) ***Basis for GRAS Determination:*** Scientific procedures.

(v) ***Statement of Availability:*** The data and information that are the basis for the GRAS notification are available for FDA review and copying at reasonable times either at Sidley Austin Brown & Wood LLP, 1501 K Street, Washington, DC 20005 or the materials can be forwarded to the FDA upon request.

Sincerely,

(b) (6)

Heather Nelson Cortes, Ph.D.

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DETERMINATION OF THE *GRAS* STATUS OF TONALIN® TG 80 FOR USE AS A FOOD INGREDIENT

EXPERT PANEL MEMBERS

Joseph Borzelleca, Ph.D., F.A.T.S

John Thomas, Ph.D., F.A.T.S.

Walter Glinsman, M.D.

DETERMINATION OF THE GRAS STATUS OF TONALIN® TG 80 FOR USE AS A FOOD INGREDIENT

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Abbreviations:

Abb.	Numeral through Letter L	Abb.	Letter L through W
5'-ND	5'-nucleotidase	LF	Low fat
ACO	Acyl-CoA oxidase	Lpa	lipoprotein (a)
ALAT	Alanine aminotransferase	LYM	Lymphocytes
ALBP	Adipocyte lipid-binding protein	M	Male
AlkP	Alkaline phosphatase	MCHC	Mean corpuscular haemoglobin concentration
ANOVA	Analysis of variance	MCV	Mean cell volume
ARS	Agricultural Research Service	MID	Microimmunodiffusion
ASAT	Aspartate aminotransferase	M/F	Males/females
BFM	Body fat mass	MPV	Mean platelet volume
BMI	Body mass index	MUFA	Monounsaturated fatty acid
CC	Piglets reared on sows fed CLA diet and then fed the CLA starter diet	NAFLD	Non-alcoholic fatty liver disease
CFR	Code of Federal Regulations	NC	Not changed
CL	Piglets reared on sows fed CLA diet and then fed the linoleic acid starter diet	ND	Unable to determine
CLA	Conjugated linoleic acid	NEFA	Non-esterified fatty acids
CLA-EE	CLA ethyl ester	NIDDM	Non-insulin dependent diabetes melitis
CLA-FFA	CLA free fatty acid	NOAEL	No observed adverse effect level
CLA-ME	CLA methyl ester	NQ	Not quantified
CLA-TG	CLA triacylglycerol	NTIS	National Technical Information Service
CO_2	Carbon dioxide	ODC	Ornithine decarboxylase
COX-2	Cyclooxygenase	OECD	Organisation Economic and Co-Operation Development
CPK	Creatine phosphokinase	OLETF	Otsuka Long-Evans Tokushima Fatty
CSFII	Continuing Survey of Food Intake by Individuals	PAFA	Priority-Based Assessment of Food Additives
CYP	Cytochrome	PCT	Procalcitonen
DR	Dietary record	PDW	Platelet distribution width
ESCODD	European Standards Committee on Oxidative DNA Damage	phip	Phenylimidazo[4,5-b]pyridine

Abb.	Numeral through Letter L	Abb.	Letter L through W
F	Female	PLT	Platelets
FABP	Fatty acid binding protein	PPAR	Peroxisome-proliferator activated receptor
FAP	Food additive petition	PPAR-γ	Peroxisome proliferator-activated receptor-g
FAT	Fatty acid transporter	PWAT	Perirenal white adipose tissue
FBS	Fetal bovine serum	PUFA	Polyunsaturated fatty acid
FCC	Food Chemical Codex	RACC	Reference amounts customarily consumed
FD	Food duplicate	RBC	Red blood cell
FDA	Food and Drug Administration	RCN	Relative cell number
FFA	Free fatty acids	RDW	Red cell distribution width
FFQ	Food frequency questionnaire	RIA	Radioimmunoassay
γ-GT	γ-Glutamyltransferase	RA	Rumenic Acid
GC/MS	Gas chromatography/mass spectrometry	SD	Sorbitol dehydrogenase
GLUT4	Glucose transporter	SFA	Saturated fatty acid
GRAS	Generally Recognized As Safe	TAG	Triacylglycerol
HCT	Hematocrit	TBA	Thiobarbituric acid
HF	High-fat	TG	Triglyceride
HGB	Hemoglobin	TNF	Tumor necrosis factor
IGF	Insulin-like growth factor	TSH	Thyroid stimulating hormone
ip	Intraperitoneal	TZD	Troglitazone
LA	Linoleic acid	USDA	United States Department of Agriculture
LC	Piglets reared on sows fed linoleic acid diet and then fed the CLA starter diet	VLCD	Very-low-calorie diet
LCLA	Linoleic acid plus CLA	WAT	White adipose tissue
LETF	Long-Evans Tokushima Fatty	WBC	White blood cell
LBM	Lean body mass	ZDF	Zucker diabetic fatty
LDL	Low-density lipoprotein		

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Abb. = abbreviation

DETERMINATION OF THE *GRAS* STATUS OF TONALIN® TG 80[1] FOR USE AS A FOOD INGREDIENT

The undersigned, an independent panel of recognized experts (hereinafter referred to as the Expert Panel[2]), qualified by their scientific training and relevant national and international experience to evaluate the safety of food ingredients, was requested by Cognis Corporation to determine the Generally Recognized As Safe (GRAS) status of Tonalin® TG 80[3] as an ingredient added to fruit juices, meal replacement products, yogurts and chocolate listed in APPENDIX A, page 122. A comprehensive search, conducted by Burdock Group, of the scientific literature for safety information on the primary constituent of Tonalin® TG 80 (*i.e.*, CLA) was initially conducted in February 2003, and continually updated thereafter, is summarized in this report. Cognis Corporation assures that all relevant, unpublished information in its possession has been supplied to Burdock Group and has been summarized in this monograph. The toxicology and safety-in-use data for Tonalin® TG 80[4] are critically evaluated. The literature search and supporting documentation were made available to the Expert Panel. In addition, the Expert Panel independently evaluated other materials deemed appropriate and necessary. Following an independent, critical evaluation, the Expert Panel conferred and unanimously agreed to the decision herein.

1. Proposed Use of Tonalin® TG 80

The proposed use of Tonalin® TG 80 is in fruit juices, meal replacement products, yogurts and chocolate milk (see APPENDIX A for complete list of foods). The proposed maximum concentration of Tonalin® TG 80 CLA in these foods is 2100 mg *per* serving (1700mg CLA/serving), except for cream substitute liquid, cream substitute powdered and chocolate milk whch is 500 mg/serving (400mg CLA/serving).

2. Description and Specifications

Conjugated linoleic acid is a naturally occurring zoochemical found primarily in dairy products and ruminant tissue. CLA is unique in that the double bonds are separated by only one single bond (Figure 1). There are eight potential isomers, but the *cis*-9, *trans*-11 (C18:2

[1] Tonalin® TG 80 is a mixture of conjugated linoleic acid (CLA) isomers (see Specifications, section 2 for more information).
[2] Modeled after that described in section 201(s) of the Federal Food, Drug and Cosmetics Act, As Amended.
[3] Tonalin® TG 80 contains 80% conjugated linoleic acid (CLA), the primary constituent.
[4] Because Tonalin® TG 80 is comprised of 80% CLA, the safety evaluation was based primarily on CLA.

*c*9,*t*11) is the most frequent naturally occurring isomer in foods. Tonalin® TG[5] 80 is a mixture of CLA[6] isomers. Tonalin® TG 80 is manufactured from safflower oil and is a mixture of 78-84% C18:2 *c*9,*t*11 and C18:2 *t*10,*c*12 CLA isomers (50:50 ratio). Specifications of Tonalin® TG 80 are presented in Table 1, which were provided by Cognis Corporation.[7] Analytical results of five samples from non-consecutive batches indicates that the Tonalin® TG 80 produced meets these specifications (see APPENDIX B, page 124).

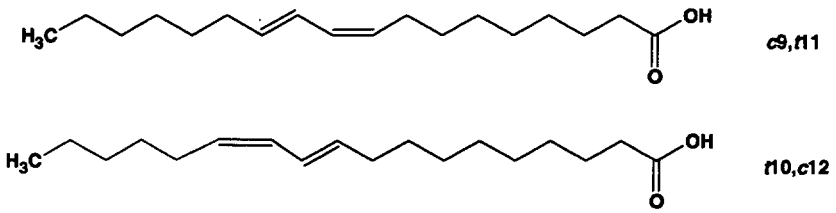

Figure 1. Chemical structure of the two primary CLA isomers of Tonalin TG 80

Table 1. Specifications for Tonalin® TG 80 (provided by Cognis Corporation)

Parameter	Specification
CLA Isomer C18:2 (*c*9,*t*11)	37.5 – 42%
CLA Isomer C18:2 (*t*10,*c*12)	37.5 – 42%
CLA Isomer C18:2 (*t*8,*c*10; *c*11,*t*13; *c,c* & *t,t*)	<2%
Palmitic Acid C16:0	<4%
Oleic acid C18:1 (c9)	11 – 14%
Stearic acid C18:0	<4%
Linoleic acid C18:2 (c9,c12)	<3%
Triglycerides	>80%
Saponification value (mg KOH/g)	185-195 (DGF C V 3)
Iodine value (g I2/100g)	≥ 115 (DGF C 5 11 A)
Peroxide value (mEq/kg)	< 10 (DGF C VI 6a)
Color	≤ 0.2 (DGF C IV 4c)
Acid value (mg KOH/g)	≤ 2 (ISO 660)
Unsaponifiable matter (w%)	≤ 1 (IUPAC 2.401)
Lead (mg/kg)	< 0.1 (USP XXII)

[5] Triglyceride.

[6] CLA refers to a group of positional and geometric isomers of the *omega*-6 essential fatty acid, linoleic acid. Linoleic acid (*cis*-9, *cis*-12, octadecadienoic acid) is a phytochemical found primarily in vegetable oils and is an 18-carbon polyunsaturated *omega*-6 fatty acid with double bonds at the *cis*-9 and *cis*-12 positions (-C=C-C-C=C).

[7] Neither Tonalin® TG 80 nor CLA are listed in the most recent edition of the Food Chemical Codex (FCC, 2003).

(b)(1)(b)()1.5aa

acid, palmitic acid, stearic acid and linoleic acid are approved food ingredients (21CFR[9]172.860; 172.862; 184.1090; 184.1065). BASF Corporation has recently filed a food additive petition (FAP) with the FDA requesting that the food additive regulations, section 573 Food Additives Permitted in Feed and Drinking Water of Animals, Title 21 of the CFR be amended "*to provide for the safe use of conjugated linoleic acid (CLA) as a source of fatty acids in swine diets at levels not to exceed 1 percent in complete feed*" (Federal Register, 2003).

4. Consumption

4.1. General explanation of consumption analysis

The purpose of this consumption analysis is to estimate total daily intake of Tonalin® TG 80[10] resulting from the proposed addition of Tonalin® TG 80 to selected foods (*i.e.*, target foods), plus intake from other background foods in which CLA occurs naturally.

To calculate the amount of Tonalin® TG 80 that is consumed from food, the concentration (mg/g) in food and the amount of food consumed (g/day) must be known. These data values are used to calculate the Tonalin® TG 80 daily intake using the equation presented in Figure 3.

Tonalin® TG 80 Intake	=	**Concentration**	x	**Food Consumption**
(mg/day)		(mg/g)		(g/day)

Figure 3. Tonalin® TG 80 daily intake equation

4.2. Consumption analysis protocol and methodologies

4.3. Sources of information used in consumption analysis

Food consumption information was obtained from the Continuing Survey of Food Intake by Individuals (CSFII) 1994-1996 database[11]. The CSFII 1994-96 data is derived from a survey in which approximately 30,000 subjects recorded the weight, brand and type of food

[9] Title 21 of the Code of Federal Regulations (CFR), 2003 Edition (http://www.access.gpo.gov/cgi-bin/cfrassemble.cgi?title=200321).
[10] Because Tonalin® TG 80 is ~80% CLA isomers, this GRAS determination is essentially for CLA with additional consideration for CLA in the consumption summary (see section 4.9, page 13).
[11] This database was obtained from the National Technical Information Service (NTIS) and produced by the Agricultural Research Service (ARS) of the United States Department of Agriculture (USDA).

eaten at each occasion over a one- or two-day period. The survey is controlled for population groups, length of survey time and other relevant factors. Weighting analysis is employed to estimate the number of individuals consuming food for the entire US population. The proposed amounts (mg/serving) of Tonalin® TG 80 to be used in the target foods were obtained from Cognis Corporation (APPENDIX A). The reference amounts customarily consumed (RACC) *per* eating occasion were used for the serving size (21CFR101.12). Consumption of CLA from background foods was derived from the published scientific literature.

4.4. Selection of target foods

Foods included in the analysis were chosen by searching the CSFII database using descriptor words to generate a list of target foods to consider for the analysis. The list of foods was evaluated by Cognis Corporation and Burdock Group, and only those foods that represented a targeted food were included. Foods known to contain CLA but not included in the analysis were those (a) for which food codes in the CSFII database were not found and (b) consisting of complex mixtures of many foods in which a CLA concentration could not be determined with sufficient certainty. Foods included in the analysis were fruit juices, meal replacement products, yogurts and chocolate listed in APPENDIX A. APPENDIX A also specifies the proposed amount (mg/g) of CLA to be added.

4.5. Concentration of CLA in target foods

The proposed amount of Tonalin® TG 80 to be added to the target foods is the maximum that would be added under this GRAS determination. This maximum amount for all food items is 2100 mg Tonalin® TG 80 *per* serving for the foods listed in APPENDIX A, except for (a) cream substitute liquid (food code 12210200) (b) cream substitute powdered (food code 12210400) and (c) chocolate milk (food codes 91705010, 91705020, 91705040, 91705050, 91705060, 91705070, 91705200, 91705300, 91705400, 91705410, 91705420 and 91770030) in which the proposed amount of Tonalin®TG 80 in these foods is 500 mg/serving.

4.6. Calculating CLA consumption from target foods

The CSFII database was searched for people that consume the selected foods. The parameters of the search included the following: (1) only individuals[12] from the years 1994-1996, (2) only individuals that reported a two-day consumption survey and (3) only individuals that consumed an amount greater than zero grams. From this dataset, Tonalin® TG 80 consumption estimates were calculated using the following procedure:

A. Cross-tabulation that produced a dataset of unique individuals consuming one or more of the selected foods.

B. Data weighting that estimated the total number of people that consumed the target foods.

C. Application of the ingredient consumption equation (Figure 3) that calculated the CLA daily intake from selected foods.

4.7. CLA consumption from background foods

Ritzenthaler *et al.* (2001) estimated the consumption of CLA in men and women using a three-day dietary record (DR) protocol and a three-day food duplicate (FD)[13] protocol[14]. Total mean CLA consumption (*i.e.*, *c*9,*t*11- and *t*10,*c*12-isomers) from background foods for men and women is presented in Table 2. CLA consumption was greater when determined by the FD method compared to the DR method, thus, consumption analysis by the DR method may underestimate the true consumption of CLA[15]. The estimated total CLA consumption from background foods for men and women is 212 and 151 mg/day, respectively. Further, these investigators also determined that the primary dietary sources of CLA are dairy products (60%[16]), beef (32%), pork (3%), poultry (2%) and other foods (3%). These findings are in agreement with other studies that evaluated CLA consumption (Herbel *et al.*, 1998; Park *et al.*, 1999).

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[12] Individuals of a certain age were not excluded from the analysis.
[13] Food duplicate protocol is a highly accurate method for estimating food ingredient consumption.
[14] Although Ens *et al.* (2001) and Fremann *et al.* (2002) reported the dietary intake of the *c*9,*t*11-CLA isomer (*i.e.*, rumenic acid), Ritzenthaler *et al.* (2001) reported total CLA consumption, which included both the *c*9,*t*11- and *t*10,*c*12-CLA isomers.
[15] This conclusion assumes that the FD method is more accurate because it is a more direct measure of CLA consumption.
[16] *Percent* of total CLA consumed.

Table 2. Total CLA daily intake from background foods (Ritzenthaler *et al.*, 2001)

	Men		Women	
	DR	FD	DR	FD
Total Mean CLA Intake (mg/day)	176	212	104	151

DR = dietary record; FD = food duplicate.

4.8. Assumptions about the appropriateness of the consumption analysis

This consumption analysis includes the following assumptions:

A. The selected foods reasonably represent the proposed sources of Tonalin® TG 80 intake as an added ingredient.

B. CLA consumption from background foods as reported in the literature is reasonably estimated (Ritzenthaler *et al.*, 2001).

The consumption analysis is considered conservative because (1) the proposed amount of Tonalin® TG 80 in these foods is maximal, (2) the higher background consumption level as reported by Ritzenthaler *et al.* (2001) was used and (3) the addition of means of daily intake from target and background foods likely over estimates total daily CLA intake[17]. Therefore, this analysis is sufficiently conservative to likely result in overestimating the true CLA daily intake.

4.9. Consumption analysis results

Based on the consumption data of the target foods, the proposed concentration of Tonalin® TG 80 in these foods and other parameters, the mean and 90th percentile consumptions of CLA are 2.72 g/day and 4.93 g/day, respectively[18]. Because Tonalin® TG 80 is composed of 80% CLA, the mean and 90th percentile estimated daily intakes of CLA from its proposed used in food are 2.18 and 3.94 g/person/day, respectively (Table 3)[19]. Of the total amount of CLA consumed from these target foods, approximately 45% would be consumed from orange juice (food code 61210220).

[17] Addition of means overestimates consumption because it assumes that all individuals eat the background foods at the same level.
[18] The estimated population of individuals to consume the target foods is 209,060,790.
[19] Assuming an average body weight of 60 kg, the mean and 90th *percentile* estimated daily intake for CLA are 30 mg/kg/day and 66 mg/kg/day, respectively.

The mean and 90th percentile CLA consumption from both its proposed use in target foods, as well as from background foods, areestimated to be 2.392 and 4.364 g/day, respectively.

Table 3. CLA daily intake (eater's only) from target and background foods

	Food Consumption (g/person/day)	
	Mean	90th Percentile
Target Foods	2.18	3.94
Background Foods	0.212	0.424†
Total*	2.39	4.36

†Calculated by multiplying the mean by 2 (DiNovi and Kuznesof, 2003).

4.10. Self-Limiting Level

CLA is not considered to have a self-limiting level, *i.e.*, the amount consumed limited by unpleasant taste, odor and/or color.

5. Biological Data

The amount of information reported in the scientific and biomedical literature regarding the biological effects of CLA is voluminous. CLA is thought to be efficacious in the management and prevention of obesity (Pariza *et al.*, 1999; Vessby and Smedman, 1999; Kelley, 2001; Mougios *et al.*, 2001; Desroches *et al.*, 2001; Thom *et al.*, 2001; Belury *et al.*, 2003), Type 2 diabetes mellitus (Belury and Vanden Heuvel, 1999; McCarty, 2000a; McCarty, 2000b) and cancer (Ip *et al.*, 1994; Ip *et al.*, 1994; Scimeca *et al.*, 1994; Belury, 1995; Ip *et al.*, 1995; Ip, 1997; Ip and Scimeca, 1997; Pariza *et al.*, 1999; Aro *et al.*, 2000; MacDonald, 2000; Kritchevsky, 2000; Futakuchi *et al.*, 2002). This critical evaluation of available data focuses on the safety of CLA in food to be consumed by humans. Throughout the rest of this document, the use of terms indicating significant differences from control group (*e.g.*, increase, decrease and reduced) were determined using statistical procedures, and so noted by multiple footnotes.

5.1. Absorption, tissue distribution, metabolism and excretion

Although studies were not found in the scientific literature that demonstrated the absorption of CLA across the human gastrointestinal tract, it is likely that it is well absorbed because it is a fatty acid. Fatty acids are extensively absorbed by the gastrointestinal tract (Feinman and Lieber, 1999; Turley, 1999). The tissue distribution and metabolism of CLA have been investigated and are described below.

5.1.1. Absorption

CLA is similar to the chemical structure of linoleic acid, and it is likely that CLA is absorbed across the human gastrointestinal tract by a similar mechanism(s) (Nilsson and Melin, 1988; Ling *et al.*, 1989; Gore *et al.*, 1994; Minich *et al.*, 1999). Linoleic acid is passively diffused across rabbit small intestinal brush border membrane vesicles (Ling *et al.*, 1989). Gore *et al.* (1994) reported that uptake of linoleic acid into isolated intestinal cells was carrier mediated.

5.1.2. Tissue distribution

Tissue distribution of CLA in rats was investigated by Sugano *et al.* (1997) and Sergiel *et al.* (2001). Sugano *et al.* (1997) demonstrated that CLA is distributed into the fatty acid portion of (in decreasing order) adipose tissue, lung, kidney, spleen, serum, liver, heart and brain. Results from this study also indicate that (a) the predominant CLA in distributed into tissues is the *c*9, *t*11-CLA isomer and (b) the *c*9, *t*11-CLA isomer is preferentially absorbed after oral administration to rats. Sergiel *et al.* (2001) demonstrated that oral administration of radiolabeled *c*9,*t*11-CLA isomer acid[20] or *t*10,*c*12-CLA is distributed (in decreasing order[21]) to the adrenals, adipose tissue, lung, liver, kidney, spleen, heart, large intestine (with feces), gastrocnemius, brain, blood, testes and stomach/small intestine. The data from these studies indicate that CLA is widely distributed throughout the body in most tissues (Sugano *et al.* 1997; Sergiel *et al.*, 2001).

5.1.3. Metabolism

In species studied, CLA is metabolized by two distinct pathways, desaturation[22] and oxidation. Desaturation of CLA has been investigated more extensively than oxidation; however, both are well-recognized metabolic pathways for CLA (Sébédio *et al.*, 2003).

CLA is desaturated by Δ6 desaturase and Δ5 desaturase (Banni *et al.*, 1996; Belury and Kempa-Steczko, 1997; Sebedio *et al.*, 1997; Liu and Belury, 1998; Sebedio *et al.*, 2001). The metabolites produced are dependent upon the type of fatty acids present in the diet

[20] *c*9,*t*11-CLA is also known as rumenic acid.
[21] Order was based on *percentage* of the administered dose *per* 100 grams of tissue.
[22] Desaturation is the removal of hydrogen atoms resulting in the addition of carbon-carbon double bonds. These enzymes also catalyze elongation of the fatty acid side chain.

(Sebedio *et al.*, 1997). For instance, when CLA is fed to rats deficient in dietary linoleic and linolenic acids, the *c*9,*t*11 and *c*10,*t*12- CLA isomers are converted into conjugated isomers of arachidonic acids (*i.e.*, 8,11,13-20:3 and 8,12,14-20:3, respectively) (Banni, 2002). These conjugated arachidonic acid-type metabolites are further desaturated into *c*5,*c*8,*c*11,*t*13-20:4 and *c*5,*c*8,*t*12,*c*14-20:4 for *c*9,*t*11 and *c*10,*t*12- CLA isomers, respectively. When rats are fed a fatty acid rich diet, the *c*9,*t*11- CLA isomer is converted into the *c*8,*c*11,*t*13-20:3 as the final end product (Banni *et al.*, 2001). The *c*10,*t*12- CLA isomer is converted into *c*6,*t*10,*c*12-18:3 and into 6,*c*10-16:2 (Banni *et al.*, 2001). CLA metabolites are incorporated into the lipid component(s)[23] of adipose tissue, liver, heart and kidney (Sébédio *et al.*, 2003).

CLA is also metabolized to carbon dioxide (CO_2) by β-oxidation *in vivo* in rats (Sergiel *et al.*, 2001). Sergiel *et al.* (2001) demonstrated that 70% of the total oral dose of *c*9,*t*11-CLA or *t*10,*c*12-CLA are converted into CO_2 over a 24-hour period. A significant proportion of CLA administered orally is oxidized to CO_2 *in vivo*.

5.1.4. Excretion

Sergiel *et al.* (2001) demonstrated that radio labeled CLA is excreted *via* expired air, urine and feces in rats (strain not specified). In this study, the primary excretory route was expired air (*i.e.*, 70% of the total dose was excreted in air) over a 24-hour period after oral administration. Urine and feces accounted for 1.3-2% and <0.5%, respectively, of the total dose. The extent to which CLA was excreted in expired air was time-dependent, reaching a plateau after 12 hours.

5.2. Biochemical Effects

Sprague-Dawley rats fed 0.02 g/kg CLA for two weeks had decreased linoleic acid in liver cardiolipin[24] compared to rats fed an equal dose of linoleic acid (Sugano *et al.* 1997). According to the investigators, this finding indicated that modified fatty acid composition in liver cardiolipin might reduce hepatic mitochondrial respiratory function. Serum and hepatic cholesterol and TBA[25] were unaffected by the CLA treatment.

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[23] Lipid components include neutral lipids and phospholipids.
[24] 1,3-Diphosphatidylglycerol, a phospholipid occurring primarily in mitochondrial inner membranes and in bacterial plasma membranes. Cardiolipin is the main antigenic component of Wassermann-type antigens used in nontreponemal serologic tests for syphilis.
[25] Thiobarbituric acid.

5.3. Summary of absorption, distribution metabolism and excretion studies

Like most fatty acids, CLA is well absorbed across the gastrointestinal mucosa. It is also widely distributed throughout the body, metabolized *via* oxidation and desaturation and extensively excreted from the body in expired air, and lesser amounts in urine and feces.

6. Toxicology studies in experimental animals

In this section, classical toxicological studies (*e.g.*, acute, subchronic, genetic, cellular and irritation/sensitization), in which experimental animals were administered CLA, are critically evaluated. Other biological effects (*i.e.*, hepatic lipid accumulation, increased plasma insulin/leptin concentration, fatty streaks, peroxisome proliferation and reduced milk fat) are also critically evaluated.

SECTION OVERVIEW

Animal Studies
6.1 – 6.7 Toxicity
Other Biological Studies
7.1 Hepatic lipids
7.2 Insulin
7.3 Aortic fat deposition
7.4 Peroxisomes
7.5 Milk fat
Human Studies
8.1 Safety
8.2 Insulin
8.3 Isoprostanes
8.4 Milk fat
8.5 Higher dose non-safety
8.6 Lower dose non-safety

6.1. Acute toxicity

6.1.1. Rats

An acute oral toxicity study in rats (strain unspecified) was performed using CLA (Tonalin®) beadlets consisting of CLA methyl ester (CLA-ME) of unknown purity (Berven *et al.* 2002). Berven *et al.* concluded that oral administration of CLA-ME to rats was found to be "*non-toxic*" based on an LD_{50} value of >2 g/kg. A higher dose was probably not tested, as *per* OECD[26] guidelines (Anonymous, 1998a).

6.2. Subchronic toxicity

6.2.1. Rats

Rats (strain not specified) were fed "*beadlet formulations*" containing 50,000 ppm or 5 g/kg day[27] (possibly lower doses as well) CLA-ME or CLA ethyl ester (CLA-EE) for an undisclosed amount of time (Berven *et al.* 2002). It was reported that "*low repeat dose toxicity for both beadlet formulations with no observed adverse effects at the highest test dose*

[26] Organization for Economic and Co-operation Development.
[27] PAFA conversation factor of 0.100 was used to convert ppm to g/kg (PAFA, 1993).

(50,000 ppm)" (Berven *et al.* 2002). Because the original report was unavailable, these findings could not be substantiated (Berven *et al.* 2002).

Scimeca (1998) investigated the subchronic toxicity of CLA[28] (1500 mg/kg/day) fed orally to adult male Fischer 344 rats for 36 weeks. At the end of the treatment period, organ weights[29], organ histopathology and blood chemistry[30] were recorded. Thymus weights (absolute and relative) were reduced[31], and adrenal weights (absolute and relative) were increased, in CLA-treated rats. No differences in mean body weights, food intake, organ weights, organ histopathology or blood chemistry was observed in the CLA treated group *versus* the control group. These data indicate that 1500 mg CLA/kg/day does not produce an overt adverse effect in the rat.

O'Hagan and Menzel (2003) recently published findings from a 90-day dose-response study in which male and female Wistar rats (*n*=20 *per* sex *per* group) were fed either a high-fat (HF) basal diet[32] or a HF basal diet containing 0%, 1%, 5% or 15%[33] Clarinol™ G80[34]. An additional control group (*n*=20 M/F[35]) was included that was fed a normal, low-fat (LF)[36], diet. Three additional recovery[37] groups (*n*=10 M/F *per* group) were fed the LF diet only (control), HF-diet only (control) and HF-diet supplemented with 15% Clarinol™ G80. The HF-diet was supplemented with 10% higher levels of protein, L-cysteine, cellulose, choline-bitartrate, minerals and vitamins to compensate for reduced food intake in rats fed a high calorie diet and maintain a normal level of nutrient intake.[38] Body weights[39], food

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[28] Specific isomeric composition of the CLA mixture included *c*9,*t*11- and *t*9,*c*11- (42.5%), *t*10,*c*12- (43%), *c*9,*c*11- (1.5%), *c*10,*c*12- (1.2%), *t*9,*t*11- and/or *t*10,*t*12- (1.8%), linoleic acid (7.1%) and unknown constituents (3.1%).

[29] Heart, thymus, spleen, adrenals, liver, kidney, stomach, small intestine, caecum, large intestine, testes and brain.

[30] White blood cell (WBC), lymphocytes (LYM), microimmunodiffusion (MID), red blood cell (RBC), hemoglobin (HGB), hematocrit (HCT), mean cell volume (MCV), mean corpuscular haemoglobin concentration (MCHC), red cell distribution width (RDW), platelets (PLT), mean platelet volume (MPV), procalcitonen (PCT), platelet distribution width (PDW).

[31] Use of relative terms (*e.g.*, increase, decrease, reduce) indicates statistical significance from either control or baseline throughout this document.

[32] High fat diet contained AIN-93G feed supplemented with safflower oil (total fat content was 15%) to ensure an equivalent caloric intake between control and treated groups.

[33] Dose of CLA in g/kg for males was 0.48, 2.4 or 7.2, and for females was 0.54, 2.7 and 8.2. Total fat content of diet for CLA treatment groups was 15%.

[34] Clarinol™ G80 contained approximately 80% CLA and will be referred to as CLA. The CLA isomer content was *c*9,*t*11- (37.3%); *t*10,*c*12- (37.6%); *c*,*c*- (1.6%); *t*,*t*- (1.3%); *c*11,*t*13- and *t*11,*c*13- (1.5%); saturated fatty acids (6.9%); monounsaturated fatty acids (12.1%) and polyunsaturated fatty acids (1.7%). Isomers were esterified with glycerol.

[35] M/F = males/females.

[36] Total fat content of LF group was 7% (safflower oil).

[37] Rats in this group were maintained on either the LF or HF basal diets for an additional 28 days (*i.e.*, four weeks), *i.e.*, 118 total days.

[38] The protocol used in this study followed OECD guidelines for repeat dose testing in 90-day oral toxicity studies in rodents (Anonymous, 1998b).

[39] Recorded once *per* week.

consumption[40] and water consumption[41] were monitored throughout the study period. Ophthalmoscopic observations were performed prior to treatment and after 87 days of treatment in rats of the control and highest dose group. On treatment days, 22 (males), 24 (females), 51 (females) and 52 (males) rats (10 from each sex/group) were fasted overnight and blood was collected for clinical chemistry analysis. Blood was also collected after the 90-day treatment period for clinical chemistry and hematology assessments. During week 13, rats (10 from each sex/group) were deprived of water for 24 hours and food for the last 16 hours. During this 16-hour period, urine was collected for volume, density and other constituents. Urine was also collected from all rats in the three recovery groups during week 17. Clinical assessments were performed weekly throughout the 90-day treatment period. During the last week of treatment, neurobehavioral[42] assessments were conducted. After completion of the treatment period, gross pathological examinations were performed, and organs[43] were removed, weighed and prepared for histopathology. Statistical procedures[44] were used to determine differences between CLA-treated and control groups.

Survival was unaffected in any of the CLA-treatment groups. Opthalmoscopic examination did not reveal any ocular changes in any of the groups. Food and water consumption were reduced 22% and 20%, respectively, in high-dose male and female rats compared to the two control groups. Lower CLA doses also appeared to reduce food consumption in male and female rats. A clear dose-response relationship between increased CLA-dose and decreased food consumption was not demonstrated. O'Hagan and Menzel (2003) proposed that the reduced food consumption in CLA-treated rats was due to "*reduced palatability.*" These investigators also reported that the

> "Reduced food intake resulted in a statistically significant reduction in body weights in high-dose male and female rats at day 7 and in high-dose female rats at day 14...This delay in

[40] Recorded "*over successive periods of 7 days.*"
[41] Recorded over four consecutive days during week 1, 6 and 12.
[42] Sensorimotor reflex, grip strength and motor activity.
[43] Organs collected included brain, epididymides, heart, kidneys, liver, ovaries, pancreas, spleen, testes, thymus, thyroid (including parathyroids) and uterus. Brown adipose tissue was assessed *in-situ* in rats treated for 90-days, and collected and weighed in three males of the HF control recovery group.
[44] Analysis of covariance followed by Dunnett's tests were used to compare body weights; one-way analysis of variance (ANOVA) followed by Dunnett's tests were used to compared food/water consumption, food efficiency, red blood cell and clotting, clinical chemistry and organ weights; Kruskal-Wallis non-parametric (ANOVA) followed by Mann-Whitney U tests were used to compare other blood cell counts and urinary parameters; Fisher's exact test was used to evaluate histopathological changes.

growth during the first weeks on study resulted in consistently lower body weights in the high-dose animals throughout the study and this effect was more pronounced in females."

Nevertheless, the effect of CLA on body weights in both male and female rats was less than 10%. CLA had no effect on food conversion efficiency. Hematology parameters were unaffected by CLA treatment.

The effects of CLA on serum liver enzymes[45] in male and female rats are presented in Table 4 and Table 5, respectively. Liver enzymes were unaffected in male rats treated with the lowest or middle CLA doses. At the highest CLA dose, liver enzymes were increased 21%-46%; however, increased liver enzymes returned to normal levels during the recovery period. The liver enzymes, AlkP and ALAT, were highest during weeks 4 and 8 of treatment and lower at week 13 in male rats. In female rats treated with the lowest and middle CLA doses, serum ALAT and 5'ND were increased 18%-36% at week 4 and/or 13, which returned to normal levels during the recovery period. Liver enzymes were increased 12%-68% in female rats treated with the highest CLA dose at weeks 4 (AlkP and ALAT only), 8 (AlkP, ALAT and 5'ND) and 13 (all five enzymes). Although serum ALAT and ASAT were increased 28% and 16%, respectively, after the four week recovery period, these enzymes were reduced compared to week 13, indicating a return to normal levels. The other three serum liver enzymes were at normal levels in female rats after the recovery period. γ-Glutamyltransferase (γ-GT) was unaffected by CLA treatment (data were not presented). Although these highest doses, 7.2 g/kg and 8.2 g/kg may have increased serum liver enzymes in male and female rats respectively, a clear dose-response relationship between CLA and serum liver enzymes is not evident.

Table 4. Effects of varying doses of CLA on serum liver enzymes in male rats (relative to control) (O'Hagan and Menzel 2003)

Dose (g/kg)	Week 4		Week 8		Week 13			Recovery		
	AlkP	ALAT	AlkP	ALAT	AlkP	ALAT	ASAT	AlkP	ALAT	ASAT
0.48	NC	NC	NC	NC	NC	NC	NC	NQ	NQ	NQ
2.4	NC	NC	NC	NC	NC	NC	NC	NQ	NQ	NQ
7.2	↑ 46%‡	↑ 38%‡	↑ 45%‡	↑ 28%‡	↑ 28%‡	↑ 26%‡	↑ 21%*	NC	NC	NC

Alkaline Phosphatase (AlkP), Alanine Aminotransferase (ALAT), Aspartate Aminotransferase (ASAT); NC = not changed relative to control group; NQ = not quantified; ‡ = significantly different from high-fat (HF) control group ($p<0.01$); * = significantly different from low-fat (LF) control group ($p<0.01$).

[45] Serum liver enzymes, *i.e.*, markers of liver damage, included aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), alkaline phosphatase (AlkP), γ-glutamyltransferase (γ-GT), sorbitol dehydrogenase (SD), 5'-nucleotidase (5'-ND).

Table 5. Effects of CLA on serum liver enzymes in female rats (relative to control) (O'Hagan and Menzel 2003)

Dose (g/kg)	AlkP	ALAT	ASAT	5'ND	SD
			Week 4		
0.54	NC	↑ 21%‡	NQ	NQ	NQ
2.7	NC	↑ 18%*	NQ	NQ	NQ
8.2	↑ 26%‡	↑ 39%‡	NQ	NQ	NQ
			Week 8		
0.54	NC	NC	NQ	NC	NQ
2.7	NC	NC	NQ	↑ 36%‡	NQ
8.2	↑ 44%‡	↑ 40%‡	NQ	↓ 42%‡	NQ
			Week 13		
0.54	NC	↑ 18%*	NC	↑ 23%*	NC
2.7	NC	NC	NC	↑ 22%*	NC
8.2	↑ 68%‡	↑ 22%*	↑ 12%*	↓ 54%‡	↑ 54%‡
			Recovery		
0.54	NQ	NQ	NQ	NQ	NQ
2.7	NQ	NQ	NQ	NQ	NQ
8.2	NC	↑ 28%*	↑ 16%*	NC	NC

Alkaline Phosphatase (AlkP), Alanine Aminotransferase (ALAT), Aspartate Aminotransferase (ASAT); 5'-Nucleotidase (5'ND); Sorbitol dehydrogenase (SD); NC = not changed relative to control group; NQ = not quantified; ‡ = significantly different from high-fat (HF) control group ($p<0.01$); * = significantly different from low-fat (LF) control group ($p<0.01$).

The effect of CLA on plasma cholesterol and triglyceride are presented in Table 6 and Table 7, respectively. The effect of CLA on plasma cholesterol appears to have been sporadic and unpredictable. For instance, in male rats, CLA was observed to increase and decrease plasma cholesterol concentrations. Further, changes in plasma cholesterol appeared to occur at only one dose in male (highest) and female (middle) rats. Plasma triglyceride levels were reduced (26-45% at all doses) in male rats and increased (65-149% at 8.2 g/kg only) in female rats. There was not a consistent dose-dependent effect on plasma triglyceride. The lack of a dose-response relationship indicates that CLA may not be directly responsible for these changes in plasma cholesterol and triglyceride concentrations.

Table 6. Effects of CLA on plasma cholesterol in male (M) and female (F) rats (relative to control) (O'Hagan and Menzel 2003)

	Week 4		Week 8		Week 13	
Dose (g/kg)	Males	Females	Males	Females	Males	Females
0.48-M 0.54-F	NC	NC	NC	NC	NC	NC
2.4-M 2.7-F	NC	↑ 38%‡	NC	↑ 17%‡	NC	↑ 17%‡
7.2-M 8.2-F	↓ 42%‡	NC	↑ 39%‡	NC	↓ 38%‡	NC

NC = not changed relative to control; NQ = not quantified; ‡ = significantly different from high-fat (HF) control group ($p<0.01$); * = significantly different from low-fat (LF) control group ($p<0.01$).

Table 7. Effects of CLA on plasma triglyceride in male (M) and female (F) rats (relative to control) (O'Hagan and Menzel 2003)

	Week 4		Week 8		Week 13	
Dose (g/kg)	Males	Females	Males	Females	Males	Females
0.48-M 0.54-F	↓ 43%*	NC	↓ 41%*	NC	↓ 36%*	NC
2.4-M 2.7-F	↓ 40%*	NC	↓ 38%*	NC	↑ 33%‡	NC
7.2-M 8.2-F	↓ 44%*	↑ 65%‡	↓ 45%*	↑ 149%‡	↓ 26%*	↑ 80%‡

NC = not changed relative to control; NQ = not quantified; ‡ = significantly different from high-fat (HF) control group ($p<0.01$); * = significantly different from low-fat (LF) control group ($p<0.01$).

The effect of CLA on blood glucose and insulin concentrations is presented in Table 8 and Table 9, respectively. CLA had little effect on blood glucose concentration. Blood glucose was unaffected in male and female rats treated with the lowest dose of CLA (0.48 and 0.54 g/kg respectively). Blood glucose was reduced 15% in female rats (2.7 g/kg) at week 4, but not at weeks 8 and 13, and unaffected in females receiving 8.2 g/kg. In male rats treated with 7.2 g/kg CLA, blood glucose was reduced 10%, 18% and 24% at weeks 8, 13 and after the four-week recovery period. Insulin concentration was unaffected in male and female rats treated with the lowest and middle CLA doses. At the highest dose of CLA (7.2 g/kg), insulin concentration was increased 133-172% in male rats, which returned to normal levels after the four-week recovery period. In female rats, insulin was increased 89% only on week 8 and not at the other time points.

Table 8. Effects of CLA on blood glucose in male (M) and female (F) rats (O'Hagan and Menzel 2003)

	Week 4		Week 8		Week 13		Recovery	
Dose (g/kg)	Males	Females	Males	Females	Males	Females	Males	Females
0.48-M 0.54-F	NC	NC	NC	NC	NC	NC	NQ	NQ
2.4-M 2.7-F	NC	↓ 15%*	↑ 15%‡	NC	NC	NC	NQ	NQ
7.2-M 8.2-F	NC	NC	↓ 10%*	NC	↓ 18%‡	NC	↓ 24%*	NQ

NC = no changed; NQ = not quantified; ‡ = significantly different from high-fat (HF) control group ($p<0.01$); * = significantly different from low-fat (LF) control group ($p<0.01$)

Table 9. Effects of CLA on blood insulin in male (M) and female (F) rats (O'Hagan and Menzel 2003)

	Week 4		Week 8		Week 13		Recovery	
Dose (g/kg)	Males	Females	Males	Females	Males	Females	Males	Females
0.48-M 0.54-F	NC	NC	NC	NC	NC	NC	NQ	NQ
2.4-M 2.7-F	NC	NC	NC	NC	NC	NC	NQ	NQ
7.2-M 8.2-F	↑ 172%‡	NC	↑ 133%‡	↑ 89%‡	NC**	↑ 133%‡	NC	NC

NC = no changed; NQ = not quantified; ‡ = significantly different from high-fat (HF) control group ($p<0.01$); * = significantly different from low-fat (LF) control group ($p<0.01$); **Increased 53% above HF control group but not statistically significant.

Organ weights (absolute and relative) were unaffected by the low CLA dose (0.48 g/kg) (Table 10). Rats treated with 2.4 g/kg CLA had increased liver weight (absolute and relative) 5%-9% in male rats, but had no effect on the weight of the other organs. At the highest dose of 7.2 g/kg, the effects of CLA on adrenal (male and female), kidney (male and female), spleen (female) and liver (female) weights were equivocal because an effect was observed when expressed as grams or grams *per* kilogram body weight, but not both. Although spleen weights and liver weights were increased 12-24% in male rats, the investigators attributed these changes to be adaptive responses, and not a consequence of an adverse effect. Histopathological examination revealed increased incidence of hepatocellular vacuolization in of male rats of HF control (16) at a low dose of 0.48 g/kg (20) groups compared to LF control (7) group. The incidence of vacuolization did not increase with increasing CLA dose. In female rats, hepatocellular vacuolization was unaffected by CLA

treatment. The amount of brown adipose tissue, evaluated *in-situ*, was reduced in the male and female rats treated with the middle and high CLA doses. Several other histopathological effects[46] were observed, but were determined to be unrelated to treatment.

Table 10. Effects of CLA on organ weights in male and female rats (O'Hagan and Menzel 2003)

	Males			Females		
Organ	0.48*	2.4*	7.2*	0.48*	2.4*	7.2*
Adrenals (g)	NC	NC	NC	NC	NC	↓ 12%*
Adrenals (g/kg)	NC	NC	↑ 15%‡	NC	NC	NC
Kidneys (g)	NC	NC	NC	NC	NC	NC
Kidneys (g/kg)	NC	NC	↑ 12%‡	NC	NC	↑ 15%‡
Spleen (g)	NC	NC	↑ 17%‡	NC	NC	NC
Spleen (g/kg)	NC	NC	↑ 24%‡	NC	NC	↑ 11%‡
Liver (g)	NC	↑ 9%‡	↑ 12%‡	NC	NC	NC
Liver (g/kg)	NC	↑ 5%‡	↑ 19%‡	NC	NC	↑ 52%‡

*Unit of dose is g/kg; NC = no changed; NQ = not quantified; ‡ = significantly different from high-fat (HF) control group ($p<0.01$); * = significantly different from low-fat (LF) control group ($p<0.01$).

These data indicate that daily consumption of CLA at 2.4 g/kg for male rats and 2.7 g/kg for female rats did not produce an adverse effect level (NOAEL) in this species. The investigators based this NOAEL on liver weights and plasma insulin concentration. The investigators also regarded this NOAEL to be "*conservative*" (O'Hagan and Menzel 2003).

6.2.2. Dogs

In dogs (strain not specified), CLA-ME beadlets (50,000 ppm or 1.25 g/kg[47]) were reported to result in "*no adverse effects*" (Berven *et al.* 2002). Consumption of beadlets containing 50,000 ppm CLA-EE was reported to result in mild liver impairment. However, histopathological examination of the liver did not reveal any untoward morphological effect. No other information on these studies was provided.

6.2.3. Pigs

It was reported in pigs (strain not specified) that pigs (strain not specified) fed CLA[48] (0, 0.48 or 0.95%) for fourteen weeks did not develop CLA induced histopathology[49] (EPL Path Report 1999). Histopathological examination revealed non-treatment related

[46] Hepatocellular hypertrophy and multifocal accumulation of alveolar macrophages in the lung.
[47] PAFA conversation factor of 0.025 was used to convert ppm to g/kg (PAFA, 1993).
[48] Isomer content was 60%.
[49] Tissues examined included brain, heart, duodenum, ileum, colon, kidneys, liver, lung, pancreas, parathyroids, pituitary, spleen, stomach, thyroids, abdominal artery, adipose tissue (periaortic, perirenal, omentum), and lymph nodes (mesenteric and cervical regions). No other parameters (*e.g.*, clinical chemistry, hematology) were reported.

morphological changes, apparently due to the method of euthanasia or agonal[50] in nature, of the brain, lung, heart and thyroid. Specifically, these alterations consisted of hemorrhage of the perivascular space of the brain (1 control, 2 fed 0.48% and 5 fed 0.95% CLA), meninges and/or parenchyma of the brain (2 fed 0.48%), perivascular space of the lung (6 control, 6 fed 0.48% and 5 fed 0.95%), alveoli (6 control, 7 fed 0.48% and 6 fed 0.95%), endocardium (5 control, 2 fed 0.48% and 3 fed 0.95%), thyroid (1 control) and lymph nodes (2 fed 0.48%).

Other lesions were reported as sporadic and unrelated to CLA treatment. These lesions included mononuclear cell infiltrates of the meninges and perivascular space of the brain (1 control), duodenum (1 fed 0.95%), periportal area of the liver (8 control, 6 fed 0.48% and 7 fed 0.95%) and stomach (4 control, 3 fed 0.48% and 2 fed 0.95%). Microscopic analysis of the kidney revealed slight to mild interstitial nephritis (7 control, 6 fed 0.48% and 7 fed 0.95%), renal cysts (2 control), interstitial fibrosis (1 fed 0.48%) and perivascular hemorrhage (1 fed 0.48%). Inflammation was detected in liver (minimal/chronic) in six control, two pigs fed 0.48% CLA and three fed 0.95% CLA[51]. Slight to mild chronic inflammation was also observed in muscularis of the ileum in one control pig. Hyperkeratosis was observed in the stomach at the junction of the esophagus with the cardiac stomach (1 control, 2 fed 0.48% and 1 fed 0.95%). Minimal surface erosion was observed in the stomach and "*pigment deposition that appeared to be hemosiderin was present in one lymph node*" of one control pig. All other tissues and organs appeared normal.

The investigators determined that the pathological lesions that appeared in both control and treated pigs were due to "*the method of euthanasia and were agonal in nature*". The investigators also determined that "*lesions in the kidney were indicative of early nephropathy, and inflammation in the liver and ileum were indicative of parasitic migration*". The investigators concluded, "*These and other spontaneous disease lesions occurred in both treated and control pigs at essentially comparable incidences and were considered to be incidental in nature*" (EPL Path Report, 1999).

[50] Relating to the process of dying or the moment of death http://216.251.241.163/semdweb/InternetSOMD/ ASP/1487248.asp.
[51] Changes in peroxisomes was not mentioned.

6.2.4. Summary - Subchronic

Subchronic toxicity studies conducted in rats, dogs and pigs have not revealed any adverse effect to supplementation of the diet with CLA. Based on the 90-day toxicity study by O'Hagan and Menzel (2003), the NOAEL for CLA in male and female rats is 2.4 and 2.7 g/kg, respectively.

6.3. Teratology and developmental studies

6.3.1. Rats

Rats (strain unspecified) were fed CLA at doses up to 1 g/kg without any sign of teratogenicity or developmental toxicity (Berven *et al.* 2002). No other information was provided.

Chin *et al.* (1994) investigated the effect of CLA[52] on neonatal development and growth in Fischer rats. Two experiments were performed. In the first experiment, female rats (8 weeks old) were mated and then fed either a basal diet[53] (control) or the basal diet supplemented with CLA (0.5% or 500 mg/kg) (n=20 *per* group). On day 10 of gestation, liver, mammary gland, skeletal muscle and abdominal adipose tissues were collected from ten rats *per* group. Fetuses were removed, weighed and examined grossly for abnormalities. The remaining rats continued to be fed the control or CLA diets throughout pregnancy. In the second experiment, the same protocol was followed as in the first experiment, except that two additional groups were included. A third group was fed the basal diet supplemented with 0.25% CLA (*i.e.*, 250 mg/kg) during gestation and lactation. A fourth group was fed the basal diet supplemented with 0.5% CLA during lactation only. Immediately after weaning, pups were fed the 0.25% CLA and 0.5% CLA diets for eight to ten weeks. Body weights and food intakes were monitored weekly.

Dietary supplementation with 0.5% CLA had no effect on maternal total food intake, body weights, mammary gland weights or liver weights. After 20 days of CLA treatment to pregnant females (*i.e.*, gestation), the CLA content of maternal liver, muscle and mammary

[52] The isomer content of CLA was not specified in this study.
[53] Basal diet contained corn oil at ~155 g *per* kilogram of feed.

gland was increased[54] 2100%, 1900% and 2300%, respectively. CLA content of fetal liver was increased 2200%. CLA uptake into maternal and fetal tissues was significant[55]. Dietary supplementation with 0.5% CLA throughout pregnancy had no effect on litter size, but increased mean pup weight (11%) and increased CLA content in breast milk (2600%)[56]. All fetuses appeared normal after gross examination (Chin *et al.*, 1994).

Dietary supplementation with 0.25% CLA[57], 0.5% CLA[58] and 0.5% CLA[59] increased CLA content in breast milk 2200%, 4800% and 4300%, respectively[60]. CLA treatment had no effect on litter size. Mean pup weights were increased 5-9% in both groups fed 0.5% CLA. Body weights were unaffected by CLA treatment in male and female neonatal pups measured between three and eight weeks of age. CLA had no effect on total food intake in male and female pups that remained on the 0.25% CLA and 0.5% CLA diets for eight to ten weeks after weaning. Total body weight gain and feed efficiency were increased 4-6% in CLA treated pups during this post-weaning period (Chin *et al.*, 1994).

These data indicated that dietary supplementation with up to 0.5% CLA was not teratogenic to rat fetuses nor produced any adverse developmental effect in rats. Instead, CLA appeared to increase body weight gain of pups from dams fed CLA.

Poulos *et al.* (2001) investigated the effect of CLA[61] fed to pregnant dams on body weight gain and body composition in Sprague-Dawley rats. On day seven of gestation, dams were fed either a basal diet (n=11) or the basal diet supplemented with CLA (0.5 g CLA *per* 100 gram of diet; n=12). Treatment continued throughout pregnancy, as well as during the entire lactation period (postnatal day 21, *a.k.a.* first day of weaning). Body weights and liver weights of dams were unaffected by CLA treatment. Litter size and weights of whole litters were also unaffected by CLA treatment. At weaning, female pups of dams fed CLA were heavier than control female pups. Male pup weights were unaffected by CLA treatment at weaning. Liver weights of male and female pups were also unaffected by CLA treatment.

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[54] Use of relative terms (*e.g.*, increase, decrease, reduce) indicates statistical significance from either control or baseline throughout this document.
[55] CLA content maternal and fetal tissues of the control group were very low (*i.e.*, < 1.5 µmol CLA *per* gram of tissue lipid).
[56] CLA content in breast milk of the control group was very low (*i.e.*, < 1.68 µmol *per* gram of tissue lipid).
[57] CLA was fed during gestation and lactation.
[58] CLA was fed during gestation and lactation.
[59] CLA was fed during lactation only.
[60] CLA content in breast milk of the control group was very low (*i.e.*, < 0.96 µmol *per* gram of tissue lipid).
[61] CLA isomers included *c*9,*t*11- (42.6%); *t*10,*c*12- (45.6%) and unidentified isomers (8.7%).

6.3.2. Pigs

Bee (2000) investigated the effect of CLA on piglet growth and tissue composition. Swiss Large White sows were fed a diet supplemented with linoleic acid[62] (n=4) or supplemented with CLA[63] (2 g/100g diet; n=6)[64] at the time of mating and throughout pregnancy and lactation. After 35 days of rearing, piglets from two randomly chosen sows were assigned to a starter diet supplemented with linoleic acid or CLA, and fed either of these diets for an additional 35 days. Body weight of each piglet was recorded at birth, weaning and after 35 days fed the supplemented starter diet (*i.e.*, post-weaning period). During the rearing period, birth weights, weaning weights and weight gain were unaffected by CLA treatment (Table 11). CLA treatment had no effect on growth in piglets reared on sows fed linoleic acid and then fed the CLA starter diet (LC). CLA treatment increased body weights, weight gain, total feed intake and carcass weights (13%-24%) in piglets reared on sows fed the CLA diet and then fed the linoleic acid starter diet (CL). Growth parameters were increased 9-17% in piglets reared on sows fed the CLA diet and then fed the CLA starter diet (CC).

Table 11. Effect of CLA (2% dietary concentration) piglet growth during the post-weaning period (Bee 2000)

Growth Parameter	LC	CL	CC
Final weight	NC	↑ 17%	↑ 09%
Weight gain	NC	↑ 24%	↑ 17%
Total feed intake	NC	↑ 22%	↑ 10%
Feed efficiency	NC	NC	NC
Carcass weight	NC	↑ 13%	NC

LC=piglets reared on sows fed linoleic acid diet and then fed the CLA starter diet. CL=piglets reared on sows fed CLA diet and then fed the linoleic acid starter diet. CC=piglets reared on sows fed CLA diet and then fed the CLA starter diet. NC=Not changed.

Bee (2000) did not report any adverse effect to piglets or sows that were exposed to CLA. The data indicated that maternal exposure to CLA enhanced growth during the weaning period (see section 7.5, page 61, that evaluates the effect of CLA on milk fat in animals).

[62] Linoleic acid contained linoleic acid (65.79%); oleic acid (23.86%); palmitic acid (5.59%); stearic acid (2.37%); linolenic acid (0.75%); behenic acid (0.42%); eicosanoic acid (0.24%); erucic acid (0.21%); myristic acid (0.14%); palmitoleic acid (0.13%).

[63] Total CLA content of supplement was ~60%. Specific CLA isomers included *c*9,*t*11- (20.33%); *t*10,*c*12- (21.73%); *c*9,*c*11- (5.59%); *c*10,*c*12- (1.33%); *t*9,*t*11-/*t*10,*t*12- (9.96%). Other fatty acid constituents included oleic acid (29.48%); palmitic acid (4.37%); linoleic acid (3.99%); stearic acid (1.67%); myristic acid (0.24%); behenic acid (0.27%); eicosanoic acid (0.18%) and palmitoleic acid (0.17%).

[64] Dose of linoleic acid and CLA were 2 grams of enriched oil *per* 100 gram of diet.

6.3.3. Summary – Teratology and Developmental

In summary, toxicity studies in rats and pigs have demonstrated that CLA has no adverse effect on the developing fetus.

6.4. Genetic toxicity

The safety-review by Berven *et al.* (2002) reports that *in vitro* and *in vivo* mutagenicity tests with CLA-ME[65] and CLA-EE[66] conducted by a manufacturer were negative. Important information regarding the methodology of these tests was not included in this report. Because the original report was not available for review, these findings could not be substantiated.

O'Hagan and Menzel (2003) studied the genotoxic effects of CLA *in vitro*, *i.e.* a bacterial mutagenicity assay and a chromosome aberration assay. In the bacterial mutagenicity assay, *Salmonella typhimurium* (strains TA98, TA100, TA102, TA1535, and TA1537) were treated with Clarinol™ G80, a mixture containing 80% CLA, at increasing concentrations from 1.6 to 5000 µg/plate (except for TA100). For TA100, the highest dose tested was 3930 µg/plate due to a calculation error; however, the investigators stated that at this concentration the solubility limit of CLA had been exceeded. Tests were conducted in the absence and presence of rat liver S9 metabolic activation system. In the chromosome aberration assay, clastogenicity was evaluated by treating human lymphocytes with CLA at concentrations between 128-300 µg/ml in absence and presence of S9 mix. Reduced cellular proliferation was observed in cultures treated with 200 µg/ml CLA or greater. CLA was found to be non-mutagenic and non-clastogenic.

6.5. Cytotoxicity

Yamasaki *et al.* (2002) investigated the dose-dependent cytotoxic effect of CLA[67], *c*9, *t*11-CLA isomer and *t*10, *c*12-CLA isomer in normal rat hepatocytes (RLN-10) *in vitro*. Cells were cultured and treated with or without CLA, *c*9, *t*11-CLA isomer or *t*10, *c*12-CLA isomer (0, 1.0, 2.5, 5.0, 10.0 or 25 µM) for 72 hours in the presence of 1% fetal bovine serum (FBS).

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[65] CLA-ME = CLA methylester.
[66] CLA-EE = CLA ethylester.
[67] CLA mixture contained the following isomers: *c*9, *t*11 (48.9%); *t*10, *c*12 (41.1%); *c*9, *c*11 and *c*10; *c*12 (3.3%); *t*9, *t*11 and *t*10, *t*12 (2.3%).

After the treatment period, cell viability was measured and expressed as relative cell number (RCN).

In this normal rat hepatocyte cell-line, both *c*9, *t*11-CLA isomer and *t*10, *c*12-CLA isomers dose-dependently reduced RCN ~30 and ~25%, respectively. The lowest concentration of *c*9, *t*11-CLA and *t*10, *c*12-CLA isomers that reduced RCN was 10 and 25 μM, respectively. The apparent threshold for *c*9, *t*11-CLA and *t*10, *c*12-CLA isomers was 5 and 10 μM, respectively. The cytotoxic concentration observed in this study was less than the cytotoxic dose (35.5-71 μM) reported by Cantwell *et al.* (1999). These data indicate that *c*9, *t*11-CLA isomer and *t*10, *c*12-CLA isomers are cytotoxic to normal rat hepatocytes when exposed to high CLA doses (*i.e.*, 10-25 μM) *in vitro*.

6.6. Irritation and sensitization toxicity

The safety-review by Berven *et al.* (2002) describes a study demonstrating CLA-ME to be non-irritating to the skin and eyes of rabbits, and non-sensitizing in guinea pigs. No other information is provided regarding methodology of this irritation study and these findings could not be substantiated.

6.7. Summary of toxicity studies

CLA is not acutely toxic orally, the oral LD_{50} exceeding 2 g/kg in the rat. A CLA dose of 7.2 g/kg for males and 8.2 g/kg for females produces a somewhat moderate increase in serum liver enzymes, indicating liver injury at very high dose (O'Hagan and Menzel 2003). It is also not mutagenic *in vitro* or an irritant *in vivo*. Data from the recent 90-day subchronic dose-response study by O'Hagan and Menzel (2003)[68] indicates that the NOAEL for CLA in male and female rats is 2.4 and 2.7 g/kg, respectively.

7. Other biological studies

A number of published and unpublished articles reported several biological effects induced by CLA in experimental animals that might be significant (DeLany *et al.*, 1999; DeLany and West, 2000; Clement *et al.*, 2002; Kelley and Erickson, 2003; Kelly, 2003). These biological effects include hepatic lipid accumulation (section 7.1), plasma insulin

[68] This is a well-designed study that controlled caloric intake, as well as maintained normal nutrient intake as recommended by OECD (Anonymous, 1998b) and FDA (Anonymous, 2003).

concentration (section 7.2), aortic fat deposition (section 7.3), peroxisome proliferation (section 7.4) and reduced milk fat (section 7.5). Each biological effect is critically evaluated to determine its significance in the safety assessment of CLA. Short discussions on the proposed mechanisms that might produce these biological effects are also presented.

7.1. Hepatic lipid accumulation

Several studies demonstrated that feeding high concentrations of CLA to mice resulted in increased hepatic lipid accumulation (Belury and Kempa-Steczko, 1997; DeLany *et al.*, 1999; Tsuboyama-Kasaoka *et al.*, 2000; Clement *et al.*, 2002). These studies are described and critically evaluated in the following paragraphs.

SECTION OVERVIEW

Animal Studies
6.1 – 6.7 Toxicity – *NOAEL 2.4/2.7 g/kg/d*
Other Biological Studies
7.1 Hepatic lipids
7.2 Insulin
7.3 Aortic fat deposition
7.4 Peroxisomes
7.5 Milk fat
Human Studies
8.1 Safety
8.2 Insulin
8.3 Isoprostanes
8.4 Milk fat
8.5 Higher dose non-safety
8.6 Lower dose non-safety

7.1.1. Critical evaluation of experimental studies – hepatic lipid accumulation

7.1.1.1. Mice

Belury and Kempa-Steczko (1997) investigated the hepatic lipid composition of CLA[69] in adult mice (Harlan Sprague-Dawley). Mice (n=12 *per* group; gender not specified) were fed a basal diet or the basal diet supplemented with CLA (0.5, 1.0 or 1.5% equivalent to 750, 1500 or 2250 mg/kg[70]) diets[71] every two days for six weeks. Weight gain, feed efficiency, lipid concentration, lipid composition[72] and liver microsome δ-6-desaturase[73] activity were measured. Weight gain was dose-dependently reduced[74] in CLA-treated mice, ~44% at 2250 mg/kg). Hepatic lipid concentration was dose-dependently increased in CLA-treated mice, with the greatest increase at 2250 mg/kg (~73%). Hepatic neutral lipids that were dose-dependently increased included *c*9,*t*11-CLA (900% at 2250 mg/kg CLA) and *t*10,*c*12-CLA (not detectable in control *versus* 0.38% at 2250 mg/kg CLA). Oleic acid (18:1) was increased 11%, but only at the highest dose of CLA (2250 mg/kg). Despite the increase in

[69] CLA contained 96% of mixed isomers, which included: *c*9,*t*11-/*t*9,*c*11-CLA (43%), *t*10,*c*12-CLA (45%), *c*9,*c*11/*c*10,*c*12-/*t*9,*t*11-/*t*10,*t*12-CLA (6%), linoleate (2%) and unknown constituents (4%).
[70] PAFA conversation factor of 1500 was used to convert *percent* to mg/kg (PAFA, 1993).
[71] Both basal and CLA diets contained 5% corn oil.
[72] Fatty acids analyzed included: 14:0, 16:0, 16:1; 9, 18:0, 18:1; 9; 18:1; 11; 18:2; 9,12 (LA), 18:2 (9,11 CLA), 18:2 (10,12 CLA), 20:1; 11, 20:4; 5,8,11,14.
[73] *delta*-6-Desaturase catalyzes the conversion of linoleic acid into arachidonic acid.
[74] Use of relative terms (*e.g.*, increase, decrease, reduce) indicates statistical significance from either control or baseline throughout this document.

hepatic lipid concentration, several fatty acids were decreased in mice treated with 2250 mg/kg CLA, which included stearic acid (27%), linoleic acid (44%) and arachidonic acid (33%). The percent conversion of linoleic acid and CLA by microsomal δ-6-desaturase was determined to be 13.3 and 11.8, respectively, in these mice. Based on these data, the authors concluded that dietary CLA intake is "*associated with increased liver lipid accumulation.*"

These dose-response data in mice suggest that CLA intake can result in increased hepatic lipid content that consists primarily of CLA, as well as a reduction in certain fatty acids, such as arachidonic acid. The authors attempted to explain the reduction in arachidonic acid by measuring the enzyme that synthesizes this fatty acid (*i.e.*, δ-6-desaturase). It was hypothesized that enzyme competition between CLA and linoleic is the mechanism by which CLA reduces hepatic arachidonic acid. Although it was demonstrated that CLA was a substrate for this enzyme in this study, neither enzyme affinity (*i.e.*, Km) nor enzyme competition assays were performed. The authors could only speculate whether linoleic acid and CLA compete for this enzyme and relied on the indirect data of increased oleic acid content (a substrate for desaturases[75]). The investigators did not report any hepatic pathology or abnormal hepatic function resulting from dietary CLA administration.

DeLany *et al.* (1999) investigated the effect of CLA[76] on body fat content in male AKR/J[77] mice. Mice were acclimated to a high-fat diet[78] for 10 days. A dose-response and a time-course study were performed in which mice were fed CLA. In the dose-response study, mice (6 weeks old) on the high-fat diet were randomly assigned to one of five treatment groups (n=12 *per* group): 0 (control), 0.25, 0.5, 0.75, or 1% CLA (*i.e.*, 0, 375, 750, 1125, 1500 mg/kg *per* day[79]) for 39 days. At the end of the treatment period, blood, selected organs[80], adipose tissues[81] and eviscerated carcass were collected and frozen. Body weights, body composition[82], adipose tissue weights (absolute), and liver and spleen (control and 1%

[75] Mazliak (1980) discusses both plant and animal desaturases that convert oleic acid into linoleic acid.
[76] Isomer content included *c*9,*t*11-CLA and *t*9,*c*11-CLA (39.1%); *t*10,*c*12-CLA (40.7%); *c*9,*c*11-CLA (1.8%); *c*10,*c*12-CLA (1.3%); *t*9,*t*11-CLA and *t*10,*t*12-CLA (1.9%); *c*9,*c*12-linoleic acid (1.1%) and unknown constituents (14.1%).
[77] AKR/J is a model for studying dietary obesity (preferential uptake of dietary carbohydrate and fat http://ajpregu.physiology.org/cgi/content/abstract/272/1/R357).
[78] *Percent* fat content "*45 kcal.*"
[79] PAFA conversation factor of 1500 was used to convert *percent* to mg/kg (PAFA, 1993).
[80] Liver, spleen, kidney, testis and heart.
[81] Adipose tissue removed included inguinal, epidymal, retroperitoneal and mesenteric.
[82] Body composition parameters included fat, protein, ash, water and adiposity index.

CLA groups only) weights (absolute) were quantified and histopathological examinations of liver and spleen were performed.

Body weights were reduced (~10%) in the highest two doses after two weeks of treatment. Kidney and testes weights were unaffected by CLA treatment; whereas, liver and spleen weights were increased 28 and 18%, respectively. Percent body fat was dose-dependently reduced (up to 35% at the highest dose of 1500 mg/kg/day); whereas percent body protein was increased (17% at 1500 mg/kg/day only). Body ash and water content were unchanged in CLA treated mice. Body adiposity index was reduced 30% at 1500 mg/kg/day and unaffected at lower doses. Weights of inguinal, epididymal, retroperitoneal and mesenteric adipose tissues were dose-dependently reduced in mice (maximum reductions occurred at the highest dose of CLA (1500 mg/kg *per* day) and were 32%, 23%, 40% and 40%, respectively). Two independent laboratory histopathological examinations reported "*minimal to moderate cytoplasmic vacuolization*" of the liver in mice fed the highest stated dose as compared to control mice. One laboratory attributed the vacuolization to lipid accumulation, while the second attributed the vacuolization to accumulation of glycogen and lipid. Histopathological analysis of the liver also revealed that the morphology of other cellular organelles appeared normal. Histopathological examination of the spleen did not reveal morphological differences between control and CLA treated mice.

DeLany *et al.* (1999) also performed a time-course study in which mice were fed either a high-fat diet (control) or a high-fat diet containing 1% CLA (*i.e.*, 1500 mg/kg/day[83]) for 12 weeks (*n*=40 *per* group). Eight mice from both groups were killed after two, four, six, eight and twelve weeks. Body weights, adipose tissue weights, body composition and liver/spleen (control and 1% CLA groups only) weights were quantified, and histopathological examinations of liver and spleen were performed. Body weights of CLA-treated mice were less than controls from week four to the end of the study. Differences in body weights between CLA-treated and control mice were the greatest at week six (17%) and the least at week twelve (<5%). Adipose tissue weights were reduced in CLA-treated mice for (1) retroperitoneal (~50%) at all time points, (2) inguinal at four (~50%), six (~60%), eight (~50%) and twelve (~50%) weeks, (3) epididymal at four (~15%), six (~50%), eight (~10%)

[83] PAFA conversation factor of 1500 was used to convert *percent* to mg/kg (PAFA, 1993).

and twelve (~10%) weeks and (4) mesenteric at six (~55%), eight (~40%) and twelve (~15%) weeks. Liver weights were increased at all time points between ~15% (at week four) to ~50% (at week 12). Spleen weights were increased (~50%) only at week eight. Percent body fat and protein were reduced 20% and 8%, respectively, after 12 weeks of CLA treatment. Body ash and water content were unaffected by CLA treatment. Body adiposity index was reduced 30% after the 12 weeks of CLA treatment. Histopathological examination revealed increased lipid deposition within the liver of CLA treated mice compared to control, whereas, in the spleen, lipid content was unaffected. The morphology of other cellular organelles in liver and spleen appeared normal.

Based on these data, the investigators concluded that CLA reduces body fat accumulation. This observation is consistent with Park *et al.*, (1997) and West *et al.*, (1998) that fed CLA (0.5%) in female ICR mice or fed CLA (1%) to male AKR/J mice, respectively. Histopathological examination by DeLany *et al.* (1999) revealed increased lipid accumulation in the liver, which is consistent with the earlier study by Belury and Kempa-Steczko (1997). Further, the increase in hepatic lipid was associated with an increase in liver weight, which was elevated throughout the twelve-week treatment period. However, it is apparent that the increase in lipid accumulation of the liver or spleen was not extensive enough to result in abnormal morphology.

Tsuboyama-Kasaoka *et al.* (2000) investigated the effect of CLA[84] on adipose tissue and liver, as well as several other organs in female C57BL/6J mice. Mice were fed a basal low-fat diet containing 11% safflower oil[85] (control group; n=14) or a low-fat diet containing 1% (*i.e.*, 1500 mg/kg) CLA[86] (CLA-treated group; n=14) for five months. At the end of the treatment period, body weights and organ weights[87] were recorded. Histologic and morphometric analyses were performed on adipose tissue and liver. Body weights of the CLA-treated group were unaffected after five months of treatment. White adipose tissue (WAT) from the parametrial, renal, retroperitoneal, abdominal subcutaneous and dorsal

[84] CLA contained the isomers *c*9,*t*11- and *t*9,*c*11- (34%), *t*10,*c*12 (36%), *c*9,*c*11- and *c*10,*c*12- (3%), *t*9,*t*11- and *t*10,*t*12 (2%).
[85] Safflower oil contained 46% oleic acid and 45% linoleic acid.
[86] Total fat content of control and CLA treated group were equal.
[87] The wet weight of white adipose tissue (parametrial, renal, retroperitoneal, abdominal subcutaneous, dorsal subcutaneous) brown adipose tissue, liver, spleen, kidney, heart and skeletal muscle (gastrocnemius and quadriceps).

subcutaneous areas was reduced 73, 93, ND[88], 96 and 97%, respectively. Brown adipose tissue weights were undeterminable because of tissue ablation[89] in the CLA-treated group. Liver and spleen weights were increased 260 and 60%; respectively. The investigators reported that enlarged livers were evident after 14 days of treatment (data was not shown). Weights of the kidney, heart and skeletal muscles were unaffected by CLA treatment. Histomorphometric analysis of white adipose tissue revealed that the diameters of adipocytes in CLA-treated mice were reduced (41%) as compared to control mice. Microscopic analysis of the liver revealed "*panlobular macrovesicular steatosis,*"[90] and no observable inflammation.

The investigators concluded, "*a dietary component causes lipodystrophy and suggests that some agents that decrease fat mass may lead to lipodystrophy*" (Tsuboyama-Kasaoka *et al.*, 2000). They also reported, "*a fat mass decrease from CLA supplementation is due to apoptosis...*" (Tsuboyama-Kasaoka *et al.*, 2000). This latter finding was based on evidence that apoptosis[91] (measured by the TUNEL assay) of adipocytes, as well as specific genes that regulate apoptosis (*i.e.*, TNF-α[92] and UCP2) were increased in CLA-treated mice.

Several factors may explain the enlarged liver observed in this study, such as mouse strain, feeding a low-fat diet and duration of the treatment. The dose of CLA administered was on the upper end of the dose-response curve (1500 mg/kg) as demonstrated in the study by DeLany *et al.* (1999). Tsuboyama-Kasaoka *et al.*, (2000) did not report that dietary CLA treatment to mice resulted in any pathological condition or altered tissue function.

Clement *et al.* (2002) investigated the effect of purified *c*9,*t*11- or *t*10,*c*12-CLA isoforms on fatty liver accumulation in female C57BL/6J mice. Mice were fed a basal diet[93] (control) or the basal diet supplemented with 0.4% (600 mg/kg[94]) *c*9,*t*11- or *t*10,*c*12-CLA isoforms[95] for four weeks (n=8). After the treatment period, WAT and livers were collected, weighed and stored at –80 degrees Celsius. Although energy intake was reduced in CLA-

[88] ND=unable to determine because of "*ablation of tissue.*"
[89] Removal of a body part or the destruction of its function, as by a surgical procedure, morbid process, or noxious substance http://216.251.241.163/semdweb/InternetSOMD/ASP/1485590.asp.
[90] Enlarged and diffuse fat containing vesicles.
[91] Apoptosis is the genetically determined destruction of cells from within due to activation of a stimulus or removal of a suppressing agent or stimulus that is postulated to exist to explain the orderly elimination of superfluous cells (http://www.m-w.com/cgi-bin/dictionary?book=Dictionary&va=apoptosis).
[92] Tumor necrosis factor-α.
[93] Basal diet contained 2.4% sunflower oil.
[94] PAFA conversation factor of 1500 was used to convert *percent* to mg/kg (PAFA, 1993).
[95] Amount of sunflower oil in CLA diets was 2%.

treated mice, body weights were unaffected by CLA treatments at the end of the four week period. WAT was reduced 85% in *t*10,*c*12-CLA-treated mice, and unaffected in *c*9,*t*11-CLA treated mice. Liver weights (reported as percent of body weight) were increased ~200% in mice treated with *t*10,*c*12-CLA, and were unaffected in *c*9,*t*11-CLA-treated mice. Hepatic lipid content was increased ~250% in *t*10,*c*12-CLA treated mice, and was unaffected in mice treated with *c*9,*t*11-CLA.

Clement *et al.*, (2002 reported, "*...mice fed a diet enriched in t10,c12-CLA (0.4% w/w) for 4 weeks developed lipoatrophy, hyperinsulinemia, and fatty liver,...*". The investigators did not demonstrate that the increased hepatic lipid content in mice was associated with any pathological disease or reduced tissue function.

7.1.2. Mechanism - hepatic lipid accumulation

Several hypotheses have been proposed that might explain the lipid accumulation in mice treated with CLA, these include. These proposed mechanisms include (1) activation of a peroxisome-proliferator activated receptor[96] (PPAR), (Moya-Camarena *et al.*, 1999) (2) increased plasma insulin and/or reduced leptin concentrations (Clement *et al.*, 2002) and (3) uptake of CLA into fat stores of the liver (Belury and Kempa-Steczko 1997).

Activation of PPAR is known to induce the transcription of proteins involved in lipid metabolism[97], transport[98] and intracellular binding[99] (Dreyer *et al.*, 1993; Kaikaus *et al.*, 1993; Keller *et al.*, 1993; Duplus and Forest, 2002; Barbier *et al.*, 2002). An early hypothesis was that the CLA induced lipid accumulation in mice was due to activation of PPAR (Moya-Camarena *et al.*, 1999). In support of the PPAR-mediated mechanism, CLA activates PPAR-γ *in vivo* and PPAR-α *in vitro* (Houseknecht *et al.*, 1998; Moya-Camarena *et al.*, 1999; Evans *et al.*, 2001; Clement *et al.*, 2002; Meadus *et al.*, 2002; Kang *et al.*, 2003; Meadus, 2003; Takahashi *et al.*, 2002; Wang and Tafuri, 2003). PPAR-γ may be the important isoform because Clement *et al.* (2002) demonstrated that the *t*10,*c*12-CLA isomer induces the expression of PPAR-γ target genes (*i.e.*, FAT/CD36 and ALBP[100]). Further, Clement *et al.*

[96] A family of intracellular proteins involved in activating gene transcription of other proteins.
[97] For example, acyl-CoA oxidase.
[98] For example, fatty acid transporter (FAT)/CD36).
[99] For example, fatty acid binding protein (FABP).
[100] Adipocyte lipid-binding protein.

(2002) hypothesized that activation of PPARs responsible for hepatic lipid accumulation is an indirect effect, rather than a direct effect of CLA, which is based on experimental data by Poirier *et al.* (2001), Peters *et al.* (2001), Yoshikawa *et al.* (2002), as well as Clement *et al.* (2002).

It is also possible that hepatic lipid accumulation is the result of increased plasma insulin and/or reduced leptin concentrations observed in rodents fed CLA (see section 7.2, page 38). For instance, Tsuboyama-Kasaoka *et al.* (2000) provided data demonstrating that hepatic lipid accumulation can be reversed by subcutaneous injection of leptin (see section 7.1.1, page 34). Alternatively, insulin, rather than leptin, may be the mediator of hepatic lipid accumulation as proposed by Clement *et al.* (2002).

7.1.3. Toxicological significance - hepatic lipid accumulation

Hepatic lipid accumulation is associated with certain pathologic states induced by xenobiotics that might result in liver injury. Diseases associated with hepatic lipid accumulation include protein malnutrition, poorly controlled adult onset diabetes mellitus, obesity, ulcerative colitis and chronic pancreatitis (Swischuk and McConnell, 1976; Castanon-Gonzalez *et al.*, 1997; Aitola *et al.*, 1998; Crabb, 1999; Miller, 2000; Chitturi and Farrell, 2001; Oldenburg and Pijl, 2001; Brunt, 2002; Clark and Diehl, 2002; Kushner, 2002; Li *et al.*, 2002; Youssef and McCullough, 2002; Roberts, 2003). Fatty liver can also be induced by several well-known xenobiotics, such as carbon tetrachloride, alcohol (chronic abuse), fialuridine and valproic acid (Kolts and Langfitt, 1984; Cunnane, 1987; Coraggio *et al.*, 1988; Barisione *et al.*, 1993; Fromenty and Pessayre, 1997). Chronic non-alcoholic fatty liver disease (NAFLD) is thought to result in liver cirrhosis and fibrosis (Chitturi and Farrell, 2001). However, it is possible that hepatic lipid accumulation observed in mice is a species-specific phenomenon that is dependent on body fat turnover (*i.e.*, rodents have higher fat turnover than humans) (Pariza *et al.*, 2001). Studies in which mice were fed CLA have not demonstrated any pathological or reduced functional consequence due to hepatic lipid accumulation. There is no evidence that hepatic lipid accumulation due to dietary supplementation with CLA observed in experimental mice is of toxicological significance. This critical evaluation of all information indicates that CLA induced hepatic lipid accumulation appears to be species specific (*i.e.*, occurs in mice only) and does not result in altered hepatic function and/or morphology.

7.2. Circulating leptin[101] and insulin concentrations

The effect of CLA on plasma leptin and insulin concentrations in experimental animals has been investigated in several studies. The following paragraphs critically evaluate these experimental studies; discuss possible mechanism(s) that might mediate altered plasma leptin and insulin concentrations, as well as the potential toxicological significance of this effect.

SECTION OVERVIEW

Animal Studies
6.1 – 6.7 Toxicity – *NOAEL 2.4/2.7 g/kg/d*
Other Biological Studies
7.1 Hepatic lipids – *no toxicological concern*
7.2 Insulin
7.3 Aortic fat deposition
7.4 Peroxisomes
7.5 Milk fat
Human Studies
8.1 Safety
8.2 Insulin
8.3 Isoprostanes
8.4 Milk fat
8.5 Higher dose non-safety
8.6 Lower dose non-safety

7.2.1. Critical evaluation of experimental studies – leptin and insulin concentrations

7.2.1.1. Mice

DeLany *et al.* (1999) described in section 7.1, page 32, investigated the effect of CLA on plasma leptin and insulin concentrations in male AKR/J mice fed a basal high-fat[102] diet (control) or the basal high-fat diet supplemented with CLA for 39 days. The investigators conducted a dose-response[103] and a time-course[104] study. Plasma leptin and insulin concentrations were measured in fasted[105] mice. In the dose-response study (*i.e.*, 0, 375, 750, 1125, 1500 mg/kg *per* day for 39 days), plasma leptin concentrations were unaffected by CLA treatment. Plasma insulin was increased ~100% at 1500 mg/kg. At the lower doses, plasma insulin appeared to increase with increases in dose, but the magnitude of change was not statistically significant from the control group. In the time-course study (*i.e.*, 1500 mg/kg for 12 weeks), plasma leptin was reduced ~70% on week 6 of treatment, but not at the other time points. Plasma insulin concentration was increased ~150 and ~160% on weeks 8 and 12; respectively, compared to the control group. Plasma insulin concentrations were unaffected by CLA at the earlier time points.

The investigators concluded that CLA resulted in a hyperinsulinemic state in mice fed a high-fat diet and the highest dose of CLA (1500 mg/kg) for 8 and 12 weeks, but not in mice fed lower doses of CLA. The investigators described this effect on plasma insulin as

[101] Stedman's Online Medical Dictionary defines leptin as "*a helical protein secreted by adipose tissue and acting on a receptor site in the ventromedial nucleus of the hypothalamus to curb appetite and increase energy expenditure as body fat stores increase.*" (http://216.251.241.163/semdweb/InternetSOMD/ASP/1533148.asp).
[102] *Percent* fat content 45 kcal.
[103] Doses of CLA were 375, 750, 1125, 1500 mg/kg, and duration of treatment was 39 days.
[104] Dose of CLA was 1500 mg/kg, and duration of treatment was 12 weeks with blood sampled on weeks 2, 4, 6, 8 and 12.
[105] Mice were deprived of food for three hours prior to sampling of blood.

"*paradoxical*" because a reduction in adipose tissue weights are usually associated with decreased plasma insulin levels (Markovic *et al.*, 1998). Further, the investigators acknowledged that the AKR/J mice strain has "*higher insulin levels and respond to a high-fat diet with higher insulin levels*" than SWR/J mice (Eberhart *et al.*, 1994; West *et al.*, 1995). The authors speculated that the biological significance of the increased plasma insulin concentration may lead to "*...mild insulin resistance.*" Further, neither tissue pathology nor reduced tissue function of any organ were reported in this obese mouse model treated with CLA.

West *et al.* (2000) investigated the effect of CLA[106] on metabolic rate[107], plasma growth hormone, insulin and glucose levels in male AKR/J mice. Prior to initiating treatment, mice were acclimated for 10 days and fed a high-fat[108] diet containing corn oil. Mice were fed either the high-fat diet only (control) or the high-fat diet containing CLA (1% or 1500 mg/kg[109]) for five weeks. Body weights, energy intake, energy expenditure[110], adipose tissue weights[111], lipid synthesis[112], plasma growth hormone, plasma insulin and blood glucose were measured. Body weights and energy intake were unaffected by CLA treatment in AKR/J mice. Inguinal, epididymal and retroperitoneal adipose tissue weights were reduced approximately 50, 40 and 80%, respectively. Mesenteric adipose tissue weights were unaffected by CLA treatment. Daytime energy expenditure was increased ~14% after two weeks of treatment, but not at other time points it was measured. Nighttime energy expenditure was increased approximately 15% after four and five weeks of treatment. Plasma growth hormone, plasma insulin and blood glucose concentrations were unaffected by the CLA treatment. *De novo* fatty acid biosynthesis was also unaffected by CLA treatment.

West *et al.* (2000) concluded that CLA reduces adipose tissue weight by increasing total metabolic rate, which is not mediated by either reduction of *de novo* fatty acid synthesis or increased uncoupling of protein gene expression. The lack of an effect on plasma insulin

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[106] Isomer content of CLA was *c*9,*t*11- and *t*9,*c*11- (39.1%), *t*10,*c*12- (40.7%), *c*9,*c*11- (1.8%), *c*10,*c*12- (1.3%), *t*9,*t*11- and *t*10,*t*12- (1.9%), linoleic acid (1.1%) and unidentified constituents (4.1%). Purity of CLA was not mentioned.

[107] Metabolic rate was assessed by measuring (1) energy expenditure and (2) uncoupling protein gene expression of skeletal muscle, white adipose tissue and kidney.

[108] 45 kcal%.

[109] PAFA conversation factor of 1500 was used to convert *percent* to mg/kg (PAFA, 1993).

[110] Day- and night-time energy expenditure was measured, by CO_2 production and O_2 consumption, prior to CLA treatment, then after 1, 2, 3, 4 and 5 weeks of treatment.

[111] Adipose tissues included inguinal, epididymal, retroperitoneal and mesenteric.

[112] *De novo* lipid synthesis was measured by incorporation of deuterum oxide into fatty acids of adipose tissue triglycerides.

concentration in this study (West *et al.*, 2000) is in contrast to the earlier study by DeLany *et al.* (1999) in which the same strain of mice were fed the same dose of CLA. The investigators explained this discrepancy in the findings between these studies by the lack of appropriate plasma sampling technique used. These investigators stated that the lack of appropriate fasting "*...likely accounts for the large variation in insulin and glucose measures and obscures true differences in insulin levels between control and CLA-treated mice*" (West *et al.*, 2000).

The study performed by Tsuboyama-Kasaoka *et al.* (2000) described in section 7.1.1, page 34, also investigated the effect of CLA on plasma leptin and insulin concentration in female C57BL/6J mice. Briefly, mice were fed a low-fat[113] control (n=14) diet (containing safflower oil) or the low-fat diet supplemented with CLA (1% or 1500 mg/kg; n=14) for five months. Throughout the treatment period, blood glucose was monitored. After eight weeks of treatment, CLA-treated mice were divided into two subgroups (n=2). One of these subgroups was infused with leptin (5 µg/day for 12 days) *via* subcutaneous implanted mini-pumps or saline. Plasma leptin concentrations were measured before and after infusion. After nine weeks of treatment, insulin tolerance[114] was tested, while glucose tolerance[115] was tested at the end of the treatment period in overnight fasted mice. Plasma insulin and leptin concentrations were measured in overnight fasted mice and non-fasted mice at the end of the treatment period.

After five months of CLA treatment, plasma insulin was increased 300% and 700% in fasted and non-fasted mice, respectively. Further, plasma leptin concentration was reduced 49% and 79% in fasted and non-fasted CLA-treated mice, respectively. Mice treated with CLA had normal blood glucose response to the glucose tolerance test. Results from insulin tolerance test revealed that in control mice, blood glucose concentration decreased 60% after 60 minutes of insulin administration. Thereafter, blood glucose concentration increased towards normal levels. In CLA-treated mice, blood glucose was moderately higher (~31%) than in control mice prior to insulin administration. After insulin was administered, blood glucose decreased ~14% and ~20% after 30 and 120 minutes, respectively. In CLA-treated mice infused with saline, plasma leptin concentration remained low (<1 ng/ml) before and

[113] Fat content of the diet was 11% in the form of safflower oil.
[114] Insulin tolerance was tested by administering 0.75 mU of human insulin.
[115] Glucose tolerance was tested by administering 1 mg/g D-glucose.

after leptin infusion, while plasma insulin concentration increased ~150%. In CLA-treated mice infused with leptin, plasma leptin concentration increased 900%, while plasma insulin concentration decreased 80%. Increasing plasma leptin concentration resulted in reduced plasma insulin in CLA treated mice.

The investigators concluded that CLA treatment of female C57BL/6J mice resulted in insulin resistance as demonstrated by the higher plasma insulin concentration of CLA-treated mice and the lack of reduction in blood glucose concentration observed in the insulin tolerance test. The investigators hypothesized that the mechanism of increased plasma insulin concentration may be due to the reduced plasma leptin concentration. However, the modulation of plasma insulin by leptin was demonstrated in only two mice. To confirm this finding, the same outcome should be reproduced in a larger group of mice. The weaknesses of this study are discussed in section 7.1.1, page 34. Importantly, this study did not demonstrate that increased plasma insulin concentration is associated with tissue pathology or reduced function in mice, because these indices were not evaluated (Tsuboyama-Kasaoka *et al.*, 2000).

Clement *et al.* (2002) investigated the effect of CLA on plasma insulin and leptin concentrations in female C57BL/6J mice. The experimental design was previously described on page 35. Briefly, mice were fed a basal diet (control) or the basal diet supplemented with *c*9,*t*11- or *t*10,*c*12-CLA isoforms (0.4% or 600 mg/kg body weight) for four weeks. At the end of the treatment period, plasma leptin and insulin, as well as blood glucose, were measured. Plasma leptin and insulin concentrations were unaffected by *c*9,*t*11-CLA treatment. However, plasma leptin concentration was reduced ~47% and plasma insulin concentration was increased ~900% in *t*10,*c*12-CLA treated mice. Neither *c*9,*t*11- or *t*10,*c*12-CLA treatments altered blood glucose concentration.

These data indicate that the *t*10,*c*12-CLA isoform, but not *c*9,*t*11-CLA, results in hyperinsulinemia in female mice. The investigators did not determine the dose-response relationship for the hyperinsulinemic effect in these mice. Lowest and maximum dose that elicits hyperinsulinemia in non-diabetic mice remains unknown. Although hepatic lipid accumulation was associated with increases in plasma insulin concentration, tissue pathology or function was not reported. The biological significance of these changes (*i.e.*, hepatic lipid accumulation and increased plasma insulin concentration) is unknown (Clement *et al.* 2002).

7.2.1.2. Rat

Houseknecht *et al.* (1998) investigated the effect of CLA[116] on plasma insulin concentrations in male Zucker rats. This strain of rat has two variants, a diabetic fatty rat[117] and lean litter mate controls[118]. Lean and fatty control rats were fed a basal diet containing 5% corn oil and 1.5% lard. For the treatment groups, fatty rats were fed either a CLA diet (5% corn oil + 1.5% CLA) or a TZD[119] diet (5% corn oil + 1.5% lard + 0.2% troglitazone[120]) for 14 days. The daily intake of CLA was 17.12 g/kg[121]. At the end of the treatment period, blood was collected and analyzed for glucose, plasma insulin and plasma free fatty acids. After 14 days of treatment, body weights in CLA fed fatty rats were similar to control and lower in the TZD fed rats. Food intake did not differ between the groups. Initial blood glucose levels were similar between the four treatment groups (lean, fatty control, fatty CLA and fatty TZD). By the end of the treatment period, blood glucose level was elevated in fatty control rats, unchanged in fatty CLA and unchanged in fatty TZD rats. Plasma insulin was elevated[122] in fatty control (~160%), fatty CLA (~950%) and fatty TZD (~200%) rats compared to lean rats. Plasma free fatty acid was unchanged in fatty control, reduced (~41%) in fatty CLA and reduced (~65%) in fatty TZD rats compared to lean rats. In the glucose tolerance test, blood glucose level increased more than ~300% in fatty control rats as compared to baseline levels. Blood glucose level increased ~180% in both fatty CLA and fatty TZD rats as compared to baseline in the glucose tolerance test.

Based on these data, the investigators concluded that "*CLA acts as an insulin sensitizing agent; normalizing glucose tolerance, improving hyperinsulinemia and lowering circulating free fatty acids, thus preventing or delaying the onset of hyperglycermia in this Zucker rat model*" (Houseknecht *et al.*, 1998). The investigators also acknowledged that after 14 days of treatment, CLA fed fatty rats were "*hyperinsulinemic*" when compared to lean rats. This study was limited by its short duration (14 days). It is also limited in that only the fatty

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[116] Administered CLA was ~90% pure with the following isomers: *c*9,*t*11- and *t*9,*c*11- (42%), *t*10,*c*12- (43.5%), *c*9,*c*11- (1%), *c*10,*c*12 (1%), *t*9,*t*11- and *t*10,*t*12- (1.5%), linoleate (0.5%), 5.5% oleate (5.5%) and unidentified constituents (5%).

[117] Zucker rat has a genetic defect in the leptin receptor that results in leptin resistance.

[118] Assumed that lean litter mate control rats are not homozygous for the genetic defect in leptin receptor.

[119] TZD=troglitazone.

[120] Troglitazone at 0.2% normalizes impaired glucose tolerance and suppresses increased blood glucose, triglycerides and free fatty acids, as well as urinary protein in Zucker fatty rats.

[121] The investigators chose this dose based on previous studies demonstrating that this dose alters hepatic peroxisome proliferation activated receptor (PPAR) gene expression.

[122] Use of relative terms (*e.g.*, increase, decrease, reduce) indicates statistical significance from either control or baseline throughout this document.

rat was treated with CLA, thus the effect of CLA on plasma insulin levels in the lean rat is unclear. Plasma leptin concentration was also not quantified. Organ histopathology and/or reduced function were not demonstrated to result from CLA treatment in the Zucker rat. (Houseknecht *et al.* 1998)

Yamasaki *et al.* (2000) investigated the effect of CLA[123] on serum leptin concentration of male Sprague-Dawley rats. Rats were fed a basal diet[124] (control) or the basal diet[125] supplemented with CLA (2% or 2 g/kg/day[126]) (*n*=16 *per* group). Body weights and food consumption were monitored throughout the study. Blood, perirenal white adipose tissue (PWAT), kidney, spleen, lung and heart were collected from four rats from each group after one, three, six and twelve weeks of treatment. Leptin concentration and lipid content of serum and PWAT tissue were determined. CLA treatment had no effect on body weights, food intake or the weights of PWAT, kidney, spleen, lung or heart. Serum triglycerides, serum phospholipids and PWAT lipid content were unaffected by CLA treatment. Serum total cholesterol was reduced[127] 35% in rats treated with CLA for three weeks, and unaffected in rats treated with CLA for one, six or twelve weeks. Serum leptin concentration was reduced 63% in rats fed CLA for one week, but unaffected in rats fed CLA for three, six or twelve weeks. Interestingly, PWAT leptin content was reduced ~21% in rats fed CLA for twelve weeks, and unaffected in rats treated with CLA for one, three or six weeks.

This study was limited to one CLA dose, thus, the dose-response relationship between CLA and serum leptin concentration cannot be determined for the Sprague-Dawley rat. Unlike other studies, plasma insulin concentrations were not measured. Although kidney, spleen, lung and heart weights were recorded, liver weights were not measured. Histological examination of the liver would have also been beneficial.

Based on the data presented, the investigators concluded that CLA reduces serum leptin concentration acutely (*i.e.*, after one week of treatment) in Sprague-Dawley rats. Unlike C57BL/6J mice, the reduction in plasma leptin concentration produced by CLA appears to be

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[123] Isomer content of CLA was *c*9,*t*11- (34.7%), *t*10,*c*12- (35.6%), *c*9,*c*11- and *c*10,*c*12- (2.3%) and *t*9,*t*11- and *t*10,*t*12- (1.6%).
[124] Basal diet contained 8% safflower oil.
[125] Basal diet supplemented with CLA contained 6% safflower oil.
[126] PAFA conversation factor of 1000 was used to convert *percent* to g/kg (PAFA, 1993).
[127] Use of relative terms (*e.g.*, increase, decrease, reduce) indicates statistical significance from either control or baseline throughout this document.

a transient effect. There might also be species differences in the leptin reducing effect of CLA. Reduced serum leptin concentration was not associated with tissue pathology and/or reduced function of the organs examined.

Rahman *et al.* (2001) investigated the effect of two forms of CLA in OLETF[128] rats, a model for non-insulin dependent diabetes melitis (NIDDM). The forms of CLA[129] used in this study were CLA free fatty acid (CLA-FFA) and CLA triacylglycerol (CLA-TG). Rats were fed a control diet containing 6.5% safflower oil or the control diet supplemented with 1% CLA (FFA or TG) for four weeks. The number of rats in each group was five or six. Normal LETF rats were also included as a control group. After the four-week treatment period, rats were fasted for 10 hours and then blood and tissues[130] were collected. Adipose tissue weights (wet) were reported, but not the weights of liver or muscle.

The initial body weights between the three OLETF groups (control, CLA-TG and CLA-FFA) were the same (*i.e.*, 137 g ±2-3) and 20% higher than normal LETO rats. At the end of the treatment period, final body weights increased 150%, 138% and 138% in OLETF control, OLETF CLA-TG and OLETF CLA-FFA rats, respectively, compared to initial body weights. The final body weights in both the OLETF CLA-treated groups were 5% lower than OLETF control rats. Final body weights in all three OLETF groups were 18-27% higher than in normal LETO control rats. Food intake was unaffected by CLA treatment in OLETF rats. Food intake of all three groups of OLETF rats was 39% greater than normal LETO control rats. Adipose tissue weights of perirenal, epididymal and omental areas were reduced 35%, 40% and 20%, respectively, in OLETF CLA-treated rats (*i.e.*, TG and FFA) as compared to OLETF control rats. Perirenal and omental adipose tissue weights of both groups of OLETF CLA-treated rats were 60-70% greater than normal LETO control rats.

Plasma leptin concentration was 43% lower in both OLETF CLA-treated groups compared to OLETF control. The plasma leptin concentration in both OLETF CLA-treated rats was 187-196% higher than in normal LETO control rats. Plasma insulin concentration

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[128] The OLETF (Otsuka Long-Evans Tokushima Fatty) rat strain is a model for "*non-insulin dependent diabetes melitis (NIDDM) that is characterized by mild obesity with visceral-fat accumulation and late-onset insulin resistance.*"

[129] CLA isoforms included *c*9,*t*11- and *t*9,*c*11- (33.2%), *t*10,*c*12- (34.2%), *c*9,*c*11- and *c*10,*c*12- (2.4%), *t*9,*t*11- and *t*10,*t*12- (1.8%), and unidentified "*fatty acid*" constituents.

[130] Brown adipose tissue (interscapular and subscapular areas), liver, perirenal adipose tissue, red gastrocnemius muscle.

was unaffected by either CLA treatment in OLETF rats. All three groups of OLETF rats had plasma insulin concentrations that were 447-665% higher than in normal LETF control rats.

The results of this study are in agreement with studies in obese rats and mice given CLA, *i.e.*, reduced adipose tissue weights, unaffected plasma insulin and reduced plasma leptin concentration. This study demonstrated no differences between feeding obese rats CLA-TG *versus* CLA-FFA. Since the investigators did not treat normal LETF rats with CLA, a direct comparison of the effects of CLA in NIDDM obese *versus* normal rats cannot be assessed by these results.

Ryder *et al.* (2001) investigated the effect of CLA on glucose tolerance, plasma hormones and metabolites and skeletal muscle insulin-dependent signaling parameters in male Zucker diabetic fatty (ZDF) rats. Two CLA formulations were used in this study, an enriched CLA that contained 90% *c*9,*t*11-isomer[131] (CLA-*c*9,*t*11) and a CLA mixture that contained 47.0% *c*9,*t*11- and 47.9% *t*10,*c*12-isomers (*i.e.*, CLA-50:50). Control rats (ZDF control) were fed a high-fat (40%) basal diet containing CLA butter (0.2% CLA concentration). CLA-treated rats were fed the high-fat basal diet plus 1.5% of either CLA-*c*9,*t*11 or CLA-50:50 for fourteen days. Percentage of fat in the CLA diets was 40%. Two other control groups were included in the study, a pair-fed control group was included that was fed the basal diet at the same level of intake as the CLA-50:50 group and a lean litter mate control group, which were fed the basal diet. Body weights, food intake and blood glucose concentration were monitored throughout the study period. After the fourteen-day treatment period, glucose tolerance[132], plasma insulin and leptin, triglycerides and non-esterified fatty acids (NEFA) were measured. Muscle and liver glycogen content, glucose transport activity of soleus muscle and other muscle enzyme activities[133] were quantified.

Body weights were unaffected by CLA-*c*9,*t*11 treatment, except on day 8 when body weights were increased 12%. In rats fed CLA-50:50, body weights were reduced 10-15% on treatment days 9 through 14. Food intake was increased 17% and 20% on treatment days 10 and 14, respectively, in the CLA-*c*9,*t*11 group. In contrast, food intake was reduced 10-30%

[131] Other isomers present in the enriched CLA included *t*10,*c*12- (0.8%), *c*7,*t*9- (4.6%), other *c*,*t*- (1.0%), other *c*,*c* (1.2%), other *t*,*t*-isomers (1.2%).
[132] D-Glucose (1g/kg) was injection *via* intraperitoneal (*ip*) into 15-hr fasted rats.
[133] Enzyme activities included glycogen synthase, tyrosine-associated phosphatidylinositol 3-kinase and Akt phosphorylation.

in the pair-fed control and CLA-50:50 groups after four days of treatment, and remained low thereafter. Blood glucose concentration was similar between the groups prior to treatment with CLA. ZDF control and CLA-*c*9,*t*11 groups had increased blood glucose concentration (~140% and ~125%, respectively) after fourteen days of treatment, indicating hyperglycemia. However, blood glucose was unchanged in lean control, pair-fed control and CLA-50:50 groups (Ryder *et al.* 2001).

Glucose tolerance tests demonstrated that all groups were glucose intolerant to varying degrees compared to the lean control group (*i.e.*, ZDF control > CLA-*c*9,*t*11 > pair fed control > CLA-50:50). For instance, glucose intolerance was the most severe in the ZDF control group (blood glucose concentrations increased approximately 200% within 30 minutes) and only somewhat less intolerant in the CLA-*c*9,*t*11 group (blood glucose increased 180%). Blood glucose concentration increased in the pair-fed control and CLA-50:50 groups 150% and 125%, respectively (Ryder *et al.* 2001). These data indicate that CLA-*c*9,*t*11 is not the CLA-isoform that attenuates the glucose intolerance status of ZDF rats.

Circulating concentrations of insulin, leptin, triglycerides and NEFA were the lowest in the lean control group (2.6 ng/ml, 1.3 ng/ml, 44.1mg/dl, 0.096 mmol/l, respectively) and highest in the ZDF control and CLA-*c*9,*t*11 groups (121.2-13.7 ng/ml, 90.1-101.2 ng/ml, 199.5-250.5 mg/dl, 0.576-0.547 mmol/l, respectively) (Ryder *et al.* 2001). In CLA-50:50 treated rats, insulin, leptin, triglycerides and NEFA were 23%, 44%, 30% and 25%, respectively, lower than the levels of these circulating substances in ZDF control. Insulin, leptin, triglycerides and NEFA concentrations in the CLA-50:50 group were 260%, 420%, 300% and 350%, respectively, compared to the lean control group. CLA does not appear to normalize circulating insulin, leptin, triglycerides and NEFA of ZDF rats, which is in agreement with the study by Houseknect *et al.* (1998). In pair-fed control rats, insulin concentration was 25% lower compared to ZDF control. Leptin and triglycerides appeared to be reduced (22% and 14%, respectively) in pair-fed control, but the changes were not statistically significant.

Muscle and liver glycogen content was unaffected by any of the dietary treatments (data was not shown) (Ryder *et al.* 2001). Observed changes in muscle glucose transport activity, as well as other muscle enzyme activities, support the finding that CLA-50:50 attenuates the glucose intolerance status of ZDF rats.

This study has the same limitation as the study by Houseknecht *et al.* (1998) that investigated the effect of CLA in Zucker rats, which is that the effect of CLA in lean control rats was not investigated. It is unknown from the results of this study whether CLA alters glucose tolerance status and circulating plasma, insulin and triglyceride concentration in the normal rat. This study also did not report that tissue pathology and/or reduced function resulting from elevated plasma insulin concentration, compared to lean control rats, in ZDF rats fed CLA, because these biological parameters were not measured (Ryder *et al.* 1998). Lastly, rats were fed a high fat diet (40%) and the investigators did not report whether the basal diet was supplemented with nutrients to maintain normal levels of intake of essential vitamins and minerals. Thus, it is unclear whether the observed changes in body weight and feed intake were due to consumption of the high fat diet alone or due to reduced nutritional intake.

7.2.1.3. Pigs

Ostrowska *et al.* (2002) investigated the effect of CLA[134] in sixteen female crossbred (Large White x Landrace) pigs. Pigs were catheterized prior to treatment for repeated blood sampling. Pigs were divided into four treatment groups (n=4 *per* group) that consisted of (1) a low-fat control diet (25 g/kg body weight), (2) a high-fat control diet (100 g/kg body weight), (3) a low-fat CLA diet (10 g/kg) and (4) a high-fat CLA diet (10 g/kg). The source of fat in the diets was palm oil, which contained an abundance of saturated fatty acids. Four days prior to initiating treatment, pigs were fed a medium fat (60 g/kg) diet every three hours. Then, pigs were fed one of the four dietary regimens described above for eight days. On treatment days 3-7, pigs were bled twice daily at 9:00 and 18:00 hours. On treatment day seven, pigs were bled every three hours for the next forty-eight hours.

Plasma NEFA concentration was increased (12-22%) in CLA-treated pigs (low-fat and high-fat diets) by the end of the treatment period. Triacylglycerol was also increased (14-18%) in CLA-treated pigs. Plasma glucose, insulin and urea were unaffected by the CLA treatments. Unlike mice, CLA does not appear to affect plasma insulin or leptin concentrations in pigs. The duration of this study was short (only one week) and means were reported without associated variance estimates (standard deviation or standard error of the

[134] CLA "*contained 55 g fatty acids as CLA per 100 g of total fatty acids.*" Specific CLA isomers were not mentioned in this report.

mean). Further, isomeric composition of administered CLA was not characterized, thus, caution is warranted when comparing these results to other studies.

7.2.2. Mechanism – leptin and insulin concentrations

There are several hypotheses that might explain the effects of CLA on plasma insulin concentrations in experimental rodent models. For instance, increased insulin sensitivity by CLA observed in rodent diabetes models (Zucker rat) may be due to activation of peroxisome proliferator-activated receptor-γ (PPAR-γ) (Houseknecht *et al.*, 1998). It has also been shown that CLA modulates PPAR in rodents and pigs (Belury *et al.*, 1997; Moya-Camarena *et al.*, 1999; Evans *et al.*, 2001; Meadus *et al.*, 2002; Takahashi *et al.*, 2002; Kang *et al.*, 2003; Meadus, 2003; Wang and Tafuri, 2003).

Alternatively, Houseknecht *et al.* (1998) proposed that

> "Non-esterified fatty acids stimulate insulin secretion with varying potencies and may be the signal for compensatory hyperinsulinemia in insulin resistant states based on data from Schoonjans *et al.* (1996) and Stein *et al.* (1997)."

Isoprostanes are also known to activate PPAR (McNamara *et al.*, 2002). While the effect of CLA on isoprostanes in experimental rodent models has not been reported in the literature, it has been reported to occur in humans that consume CLA (see section 8.3, page 83). Further work in this area might be warranted.

Unlike the Zucker rat, the AKR/J mouse model does not have a genetic defect that models diabetes. Rather, the AKR/J mouse has a propensity to become obese. In this mouse model, DeLany *et al.* (1999) showed dietary intake of CLA can result in increased plasma insulin concentration. Increased plasma insulin concentration was also observed in three other mouse studies, one that used AKR/J mice and two that used C57BL/6J mice (Tsuboyama-Kasaoka *et al.*, 2000; West *et al.*, 2000; Clement *et al.*, 2002). DeLány *et al.* (1999) referred to the differential effect of CLA in the diabetic Zucker rat and the obese AKR/J mouse to be "*paradoxical.*" These investigators proposed the following hypothesis to explain this apparent paradox of CLA treatment on plasma insulin.

> "The apparent paradox in the study reported here might be explained by the milder degree of obesity in the dietary obese mouse model compared with the Zucker fatty rat and the reported effects of CLA to stimulate lipolysis in adipocytes (Park *et al.*, 1997). If CLA induces a mild

> chronic lipolytic state such that fatty acids are more readily available to skeletal muscle, this could lead to a mild insulin resistance. Elevations of fatty acids are known to lead to impaired insulin-stimulated glucose disposal, perhaps through the glucose fatty acid cycle (Randle *et al.*, 1963). Theoretically, the greater availability of fatty acids for oxidation inhibits glucose oxidation, which may be due to a reduced glucose uptake in muscle and in the whole body (Thiebaud *et al.*, 1982; Hardy *et al.*, 1991). Fatty acids may also prime the β-cell for glucose-stimulated insulin secretion (Opara *et al.*, 1992; Chen *et al.*, 1994; Stein *et al.*, 1996).
>
> Therefore, the moderate hyperinsulinemia induced by CLA could be attributable to effects on both glucose utilization, resulting in mild peripheral insulin resistance, and CLA, having direct stimulatory effects on islet insulin release." (DeLany *et al.*, 1999)

Experimental evidence supporting this hypothetical mechanism for the observed increased plasma insulin concentration of non-diabetic mouse models has yet to be generated and reported in the scientific literature.

Tsuboyama-Kasaoka *et al.* (2000) proposed that the observed increase in plasma insulin concentration and insulin resistance may be due to (1) leptin deficiency and/or (2) direct induction of TNF-α by CLA. It has also been shown that leptin enhances insulin-mediated stimulation of glucose disposal (Kamohara *et al.*, 1997; Cusin *et al.*, 1998; Ogawa *et al.*, 1999; Shimomura *et al.*, 1999). In theory, reduction in plasma leptin concentration may partly mediate an increase in insulin resistance. Alternatively, induction of TNF-α may also lead to increased insulin resistance because TNF-α is reported to decrease GLUT4[135] gene expression (Stephens and Pekala, 1991). In support of this hypothesis, Tsuboyama-Kasaoka *et al.* (2000) showed increased TNF-α and reduced GLUT4 gene expression of CLA treated mice.

Lastly, experimental data by Ryder *et al.* (2001) suggests that altered blood glucose, plasma insulin and plasma leptin concentrations observed in the Zucker diabetic rat may be partially reduced by decreased food intake. Additional studies are needed to confirm the role of food intake on modulating circulating glucose, insulin and leptin concentrations.

[135] GLUT4 has been shown to enhance glucose disposal and transport into adipocytes in transgenic mice (Shepherd *et al.*, 1993).

7.2.3. Toxicological significance – leptin and insulin concentrations

Numerous review articles have been published that discuss possible pathologies that might result from a hyperinsulinemic state, or insulin resistance (Russell, 2001; Deedwania, 2003; Grant, 2003; Hamaguchi and Namba, 2003; Hsueh and Law, 2003; Hsueh and Quinones, 2003; Hsueh, 2003; Miller, 2003; Reaven, 2003; Reaven and Tsao, 2003; Seely and Solomon, 2003; Wheatcroft *et al.*, 2003). Insulin resistance is suspected to play a role in mediating hypertension induced cardiovascular disease, atherogenic dyslipidemia (*i.e.*, atherosclerosis), abdominal obesity and impaired hemostasis. Of these, cardiovascular disease is of greatest concern, because it is a leading contributor to morbidity and mortality (Deedwania, 2003). However, Jilma *et al.* (2000) investigated the relationship between plasma insulin and (a) circulating adhesion molecules (*i.e.*, cICAM-1, vascular cell adhesion molecule-1 and circulating E-selectin)[136] and (b) other endothelial markers (*i.e.*, von Willebrand factor and soluble thrombomodulin) in human males. A relationship between increased plasma insulin and circulating adhesion molecules or endothelial markers was not found. Kessler *et al.* (2001) also found no relationship between plasma insulin and von Willebrand factor in humans or cultured endothelial cells.

The effect of CLA on circulating insulin and leptin concentrations has been investigated in a number of rodent studies. However, it is difficult to obtain a consensus from the data generated, because the effect of CLA appears to vary depending on several factors, such as: whether the animal model is predisposed to develop diabetes, susceptible to obesity, the dietary fat content and whether the animals were fasted prior to sampling of blood. Only three studies investigated the effect of CLA on plasma insulin concentration in normal mice or rats (Tsuboyama-Kasaoka *et al.*, 2000; Yamasaki *et al.*, 2000; and Clement *et al.*, 2002). Studies by Tsuboyama-Kasaoka *et al.* (2000) Clement (2002) reported similar effects on plasma insulin by CLA treatment.

Clement *et al.* (2002) proposed that increased plasma insulin concentration in non-diabetic mice mediates hepatic lipid accumulation. This hypothesis is based upon several pieces of experimental evidence. For instance, insulin is known to induce the expression of PPAR-γ, which in turn, induces the expression of FAT/CD36 and ALBP (Vidal-Puig *et al.*,

[136] Circulating adhesion molecules are believed to be potential risk factor for cardiovascular disease.

1997; Clement *et al.*, 2002). Further, hyperinsulinemia is associated with increased hepatic PPAR-γ gene expression and liver steatosis in mice (Boelsterli and Bedoucha, 2002). It is possible that increased plasma insulin concentration mediates the observed hepatic lipid accumulation observed in mice. However, it must be remembered that CLA induced hepatic lipid accumulation has not been demonstrated to result in any tissue pathology and/or reduced tissue function in experimental animals. In absence of any evidence of an adverse effect, the effect of CLA on plasma insulin concentration is of no toxicological concern.

7.3. Aortic Fat Deposition

SECTION OVERVIEW

Animal Studies
6.1 – 6.7 Toxicity – *NOAEL 2.4/2.7 g/kg/d*
Other Biological Studies
7.1 Hepatic lipids – *no toxicological concern*
7.2 Insulin – *no toxicological concern*
7.3 Aortic fat deposition
7.4 Peroxisomes
7.5 Milk fat
Human Studies
8.1 Safety
8.2 Insulin
8.3 Isoprostanes
8.4 Milk fat
8.5 Higher dose non-safety
8.6 Lower dose non-safety

In numerous studies[137], CLA has been reported to have an anti-atherosclerotic (*i.e.*, anti-aortic fat deposition) effect in experimental animals fed CLA. In contrast, Munday *et al.* (1999) has reported that CLA might result in fatty streak formation in mice. Because chronic fatty streak formation is a pathological condition that plays a role in the development of atherosclerosis, and eventually cardiovascular disease, the study by Munday *et al.* (1999) is critically evaluated in the following paragraphs.

7.3.1. Critical evaluation of experimental studies- fatty streaks

7.3.1.1. Mice

Munday *et al.* (1999) investigated the effect of CLA[138] on 'fatty streak' formation in female C57BL/6 mice. Mice were fed either a high-fat diet[139] (control), high-fat diet supplemented with 2.5 g/kg[140] (*i.e.*, 0.375 g/kg body weight[141]) CLA[142] or high-fat diet supplemented with 5 g/kg (*i.e.*, 0.75 g/kg body weight) CLA[143] for 15 weeks. At the end of the treatment period, mice were fasted for 8-12 hours; blood was then collected by cardiac puncture and liver sampled for histological examination. Body weights, food intake, mean

[137] Lee *et al.*, 1994; Munday *et al.*, 1999; Nicolosi *et al.*, 1997; Kritchevsky *et al.*, 2000; Brown and McIntosh, 2003; Nagao *et al.*, 2003; Toomey *et al.*, 2003.
[138] CLA solution contained >95% CLA; however, specific isomers were not characterized.
[139] High-fat diet contained 5 g/kg linoleic acid, maize oil (45 g/kg), olive oil (50 g/kg), anhydrous milk fat (50 g/kg) and cholesterol (10 g/kg).
[140] Kilograms (kg) refers to feed, not body weight.
[141] PAFA conversation factor of 0.15 was used to convert ppm to mg/kg body weight (PAFA, 1993).
[142] Diet also included 2.5 g/kg linoleic acid, as well as the other fat containing components.
[143] No linoleic acid was added to this diet as well as the other fat containing components.

serum total cholesterol concentration and mean serum HDL-cholesterol concentration were unaffected by CLA treatment. Mean serum HDL-cholesterol to total cholesterol ratio and mean serum triglycerylglycerol concentrations were increased[144] 26% and reduced 16%, respectively, in mice fed 0.75 g/kg CLA. Serum HDL-cholesterol to total cholesterol ratio and mean serum triglycerylglycerol concentrations were unaffected in mice fed 0.375 g/kg CLA. Mean total aortic fatty streak area was increased 150% in mice fed 0.375 g/kg CLA and unaffected in mice fed the higher dose of 0.75 g/kg CLA.

Based on the plasma HDL-cholesterol and triglycerylglycerol data, the investigators concluded that CLA treatment produced a lipoprotein profile that is indicative of reduced atherogenic potential. Fatty streak formation increased in CLA-treated mice, which, according to the investigators, might indicate a pro-fatty streak effect by CLA. This finding is in stark contrast to several other studies demonstrating an anti-fatty streak effect in hamsters, rabbits, transgenic apoE-/- mouse and rats (Lee *et al.*, 1994; Nicolosi *et al.*, 1997; Kritchevsky *et al.*, 2000; Nagao *et al.*, 2003; Toomey *et al.*, 2003).

7.3.2. Mechanism – aortic fat deposition

The mechanism of fatty streak progression is an important consideration because hypotheses exist that might explain the anti-fatty streak and pro-fatty streak effects of CLA. For instance, it is believed that the anti-fatty streak effect might result from activation of PPAR[145] (Duval *et al.*, 2002; Francis *et al.*, 2003; Puddu *et al.*, 2003). The proposed hypothesis is that activation of PPAR limits vascular inflammation, thus preventing the development of fatty streaks and atherosclerosis. In numerous studies, it has been demonstrated that CLA modulates PPAR (Belury *et al.*, 1997; Moya-Camarena *et al.*, 1999; Evans *et al.*, 2001; Clement *et al.*, 2002; Meadus *et al.*, 2002; Takahashi *et al.*, 2002; Kang *et al.*, 2003; Meadus, 2003). Studies by Meadus (2003), Clement *et al.* (2002), Yu *et al.* (2002), Meadus *et al.* (2002) and Evans *et al.* (2001) report either PPAR activation by CLA, increase in PPAR gene transcription/expression or increase in PPAR protein *in vivo* and/or *in vitro*. However, studies by McNeel and Mersmann (2003), Kang *et al.* (2003), Takahashi *et al.* (2002), and Evans *et al.* (2001) report no increase in PPAR gene expression or PPAR protein

[144] Use of relative terms (*e.g.*, increase, decrease, reduce) indicates statistical significance from either control or baseline throughout this document.

[145] The mechanism of anti-atherosclerosis effect by PPAR is *via* modulation of inflammation.

levels *in vivo* and/or *in vitro*. Collectively, the data from these studies indicate that the effect of CLA on PPAR is complex, and that the effect is likely dependent on (1) species differences (*e.g.*, pig, rat, mouse), (2) tissue differences, (3) PPAR isoform (*e.g.*, α *versus* γ) and (4) CLA isoform (*c*9,*t*11- *versus* *t*10,*c*12-) administered. Further, other mechanism(s) may play a role in CLA anti-fatty streak effect, such as COX inhibition (Toomey *et al.*, 2003).

In theory, fatty streaks may result from the insulin resistant state observed in experimental animals fed CLA (see section 7.2, page 38). Insulin resistance is hypothesized to be a potential mediator of fatty streak development (Deedwania, 2003; Grant, 2003; Hamaguchi and Namba, 2003; Hsueh and Law, 2003; Hsueh and Quinones, 2003; Hsueh, 2003; Reaven and Tsao, 2003; Wheatcroft *et al.*, 2003). Experimental evidence indicates that the mechanism by which CLA induces hyperinsulinemia in mice is mediated by modulation of PPAR (Clement *et al.*, 2002). It is conceivable that CLA modulates PPAR resulting in hyperinsulinemia, leading to an insulin resistant state and eventually fatty streaks in certain species.

7.3.3. Toxicological significance – aortic fat deposition

The evidence is insufficient to conclusively determine that CLA induces fatty streaks in experimental animals because this effect is reported in only one study, and there are several other studies demonstrating an anti-fatty streak effect in experimental animals. The association between hyperinsulinemia observed in experimental rodent models and the development of fatty streaks is speculative. Based on this single report, the effect of CLA on inducing fatty streaks *in vivo* is equivocal.

7.4. Peroxisomal proliferation

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Conjugated linoleic acid induces hepatic peroxisomal proliferation in experimental animals (Belury *et al.*, 1997; De Deckere *et al.*, 1999; Jones *et al.*, 1999; Moya-Camarena *et al.*, 1999). Peroxisomal proliferation may have toxicological significance because peroxisomal proliferators are considered non-genotoxic hepatocarcinogens in rodents (Roberts, 1999). The results from these studies are presented in Table 12 and critically evaluated in the following section.

Table 12. Summary of the effect of CLA on peroxisomal indices

Species/Strain	M/F	CLA Dose (g/kg)	Route	Duration	Peroxisomal Indices	Reference
Mice/SENCAR	F	0.75, 1.5, 2.250 – CLA mixture	Oral	6 weeks	↑ mRNA	Belury *et al.* (1997)
Hamster/F_1B	M	6 – CLA mixture 5.6 - *c*9,*t*11-CLA* 4.9 - *t*10,*c*12-CLA*	Oral	8 weeks	Unaffected	de Decker *et al.* (1999)
Rat/Wistar	M	2.5 – CLA mixture	Oral	4 weeks	Unaffected	Jones *et al.* (1999)
Rat/Sprague-Dawley	M/F	0.5, 1.0, 1.5 – F 1.5 - M	Oral	6 weeks	No correlation between indices and peroxisome number	Moya-Camarena *et al.* (1999)

M = male; F = female; *Total CLA (*c*9,*t*11- and *t*10,*c*12-isomers) was 6 g/kg

7.4.1. Critical evaluation of experimental studies - peroxisomal proliferation

7.4.1.1. Mice

Belury *et al.* (1997) investigated the effect of CLA[146] on several hepatic indicators of peroxisomal proliferation[147] in female SENCAR mice. Mice were fed a basal diet[148] (control), or the basal diet supplemented with 0.5, 1.0, or 1.5% CLA (*i.e.*, 750, 1500 or 2250 mg/kg) every other day for six weeks (n=12 *per* group). Body weights were monitored throughout the study. After the six-week treatment period, livers were removed and analyzed for hepatic lipid composition (Table 12).

[146] Isomers in administered CLA were *c*9,*c*11- and *t*9,*c*11- (43%), *t*10,*c*12- (45%), *c*9,*c*11-, *c*10,*c*12-, *t*9,*t*11-, *t*10,*t*12- (6%), linoleate (2%) and unidentified constituents (4%).
[147] Peroxisomal proliferation indicators measured were mRNA expression of acyl-CoA oxidase (ACO), cytochrome P4504A1 (CYP4A1), and liver fatty acid binding protein (FABP), as well as ornithine decarboxylase (ODC) activity.
[148] Basal diet contained 5% corn oil, which contained oleate (25%), linoleate (56%) and CLA (0.17%).

Compared to control, body weights were reduced[149] 8%, 14% and 14% in mice treated with 0.5%, 1.0% or 1.5% CLA, respectively. Total hepatic lipid concentration was increased 28%, 46%, and 81%, respectively, in mice treated with 0.5%, 1.0% or 1.5% CLA, respectively. Hepatic content (mg/g tissue) of CLA (*c*9,*t*11- and *t*10,*c*12- isomers) was below detection in the control group and 0.67 (0.5% CLA), 0.94 (1.0% CLA) and 1.06 (1.5% CLA). Hepatic linoleic acid content decreased 23% and 42% in 1.0% CLA- and 1.5% CLA-treated groups, respectively. Hepatic arachidonic acid content also decreased 63% in the 1.5% CLA-treated group. Hepatic arachidonic acid content appeared to be reduced in the 0.5% CLA- and 1.0% CLA-treatment groups, but the difference from the mean was not statistically significant. Hepatic mRNA expression of ACO, CYP4A1 and FABP were dose dependently increased with the maximum occurring in mice fed 1.0% CLA. Although ACO, CYP4A1 and FABP mRNA levels were increased in the 1.5% CLA group compared to control, expression of these proteins was lower relative to the 1.0% CLA group. ODC activity was increased, relative to control, in mice fed 1.0% CLA (~700%) and 1.5% CLA (~800%) groups.

Belury *et al.* (1997) report, "*These studies are the first to demonstrate that increasing levels of dietary CLA induce peroxisome-associated enzyme accumulation. These data suggest not only a mechanism of action of the beneficial effects of CLA (i.e., chemoprevention in extrahepatic tissues) but also predict some negative effects of this dietary fatty acid (i.e., increased risk for liver tumor promotion).*" However, this study has several limitations because only indirect measures of peroxisome proliferation[150] were quantified, rather than evaluating peroxisome number by microscopy. Liver weights were not reported and histopathological analysis was not performed. It is unknown whether CLA increased hepatic lipid accumulation.

7.4.1.2. Rats

Jones *et al.* (1999) investigated the effect of CLA[151] on peroxisome proliferation[152] in male Wistar rats (Table 12). Prior to initiating treatment, all rats were fed a basal diet[153] for

[149] Use of relative terms (*e.g.*, increase, decrease, reduce) indicates statistical significance from either control or baseline throughout this document.

[150] Indirect measures of peroxisomal proliferation were mRNA of ACO, CYP4A1 and FABP and ODC.

[151] Isomers in administered CLA were *t*9,*c*11- and *c*9,*t*11- (14.3%), *t*10,*c*12- (14.3%), *c,c*- (0.7%), *t,t*- (0.8%) and unidentified constituents (0.3%). Specific isomers in the *c,c* and *t,t* fractions were not reported. Methodology was followed that incorporated the CLA isomer mixture into triglyceride.

[152] Peroxisome proliferation indices included cyanide-insensitive palmitoyl coenzyme A (PCoA), oxidase and carnitine acetyl transferase (CAT) assays, and total cytochrome P450 of liver microsomes.

one week. Then, rats were fed a basal diet (control), basal diet containing 0.75% CLA or the basal diet containing 2.5% (*i.e.*, 2.5 g/kg[154]) CLA for four weeks (*n*=7 *per* group). A positive control group was also included that were fed the basal diet for four weeks, then at the end of the treatment period, rats were administered clofibrate in corn oil (250 mg/kg) by oral gavage for four days. Body weights and body weight gain were monitored throughout the study. After completion of the four-week treatment period, the liver and heart were collected and weighed. Body weights and body weight gain were unaffected throughout the study by CLA treatment. Clofibrate treatment increased liver weights 30% when measured as grams or 41% when measured as relative to body weights (units were not reported). Heart weights were not reported of rats treated with clofibrate. The effect of CLA on food intake was not reported. CLA treatment also had no effect on PCoA oxidase activity, CAT activity or total cytochrome P450 content. Clofibrate increased PCoA oxidase activity (480%), CAT activity (2,180%) and total cytochrome P450 content (65%).

These investigators concluded that CLA does not induce hepatic peroxisome proliferation in the rat. Further, the authors speculated that there might be species differences between the mouse and rat in response to increases in peroxisome proliferation by CLA. Alternatively, the lack of an effect on peroxisome proliferation observed in this study may be explained by the low dose of CLA (2.5 g/kg) administered and the shorter duration of treatment. The isomer composition of CLA used in this study was approximately 30% less than the isomer composition used by Belury *et al.* (1997). Further, a limited amount of data was presented in this study by Jones *et al.* (1999). For instance, liver histopathology and liver lipid content were not reported, which are frequently assessed and would have been useful for comparison to other studies. Although the data may suggest that CLA does not induce peroxisome proliferation in the rat, the use of a CLA mixture with a low isomer content and lack of supporting data leaves the possibility that the lack of an effect in this study could have been dose related (*i.e.*, the dose was insufficient to elicit a response).

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[153] The basal diet contained 13.1 g/100 g feed of fat, 0.01 g/100g of cholesterol, as well as oleic acid (33.6-35.4% of total fat) and linoleic acid (10-23%).

[154] PAFA conversation factor of 1000 was used to convert *percent* to mg/kg body weight (PAFA, 1993).

Moya-Camarena *et al.* (1999) investigated the effect of CLA[155] on peroxisome proliferation in male and female Sprague-Dawley rats. Rats were fed a basal diet[156] or the basal diet containing CLA (0.5%, 1.0% or 1.5%[157]; *n*=5 *per* group) for six weeks. Female rats were fed all three doses of CLA, whereas male rats were fed the highest CLA dose. Body weights and food consumption were monitored weekly. At the end of the treatment period, livers were removed, weighed, frozen in liquid nitrogen and stored at –80 degrees Celsius. Total hepatic lipid content, hepatic fatty acid composition[158], peroxisome proliferation indices[159] were quantified, and livers examined histologically for peroxisomes.

Body weights, food intake and liver weights were unaffected by CLA treatment in male and female rats. CLA (1500 mg/kg) increased hepatic lipid content ~6% and ~9% in male and female rats, respectively. CLA isomers in livers of male and female control rats fed the basal diet (*i.e.*, 0% CLA) contained *t*9,*c*11-CLA, *t*9,*t*11-CLA and *c*10,*t*12-CLA isomers between 0.03-0.22% of total fatty acids. In contrast, hepatic content of *c*9,*c*11-CLA was non-detectable in male and female rats fed the basal diet. Hepatic CLA content increased between 0.21-1.64% of total fatty acids for all four CLA isomers in male and female rats fed the highest dose of CLA (1500 mg/kg). Significant hepatic accumulation of CLA occurred in rats fed CLA for six weeks (Table 12).

The most abundant fatty acid was 18:0 (14.67-21.35% of total fatty acids) in rats fed the basal diet, which increased 25% in female rats fed the highest CLA dose of 1500 mg/kg, and unaffected in male rats fed an equally high dose of CLA. In female rats fed the highest CLA dose, the phospholipid fraction of fatty acids 14:0, 16:1, 18:1 *n*7, 18:2 *n*6 and 20:1 *n*9 were reduced 84%, 55%, 38%, 26% and 49%, respectively. In contrast, 20:1 *n*9 was the only phospholipid fatty acid that was reduced (40%) in male rats fed the highest CLA dose. These data indicate that CLA has differing effects on the hepatic phospholipid fatty acid content in female *versus* male rats.

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[155] Isomers in administered CLA were *t*9,*c*11- and *c*9,*t*11- (43%), *c*10,*t*12- (45%), *t*9,*t*11- , *t*10,*t*12- , *c*9,*c*11- , *c*10,*c*12- (6%), linoleic acid (2%) and unidentified constituents(4%).

[156] All diets contained 5% corn oil (2.8% linoleic acid) and a total fat content of 6% (cocoa butter).

[157] 500, 1000 or 1500 mg/kg; PAFA conversation factor of 1000 was used to convert *percent* to mg/kg body weight (PAFA, 1993).

[158] Fatty acids analyzed were 14:0, 16:0, 16:1 *n*7, 18:0, 18:1 *n*7, 18:2 *n*6, 18:3 *n*6, *t*9,*c*11-CLA, *c*9,*c*11-CLA, *t*9,*t*11-CLA, *c*10,*t*12-CLA, 20:1 *n*9, 20:4 *n*6, 20:5 *n*3.

[159] Peroxisome proliferation indices included acetyl-CoA oxidase (ACO), fatty acid binding protein (FABP) and cytochrome P-450 4A1 (CYP4A1) hepatic mRNA.

Of the peroxisome proliferation indices measured, ACO and FABP mRNA were increased (180% and 100%, respectively) in male rats fed 1500 mg/kg CLA. In female rats, ACO mRNA was unaffected by CLA treatment, whereas FABP mRNA was increased 55% and 90% in 500 mg/kg and 1000 mg/kg CLA treated female rats, respectively. Hepatic FABP mRNA was unaffected in female rats fed 1500 mg/kg CLA. Hepatic CYP4A1 was unaffected by CLA treatment in both male and female rats. Histological examination of livers from male rats fed the highest dose of CLA (1500 mg/kg), revealed no effect on the number of peroxisomes. These data indicate that in male rats fed CLA, increases in hepatic ACO and FABP mRNA are not a reliable indicator of peroxisome proliferation *in vivo*.

There are experimental deficiencies in the study by Moya-Camarena *et al.* (1999), such as lack of characterization of CLA isomer, only one dose (*i.e.*, 1.5% or 1500 mg/kg) administered to male rats (females received three doses) and lack of histopathology of rat female liver.

In Sprague-Dawley rats, "*CLA did not have a severe effect on hepatic lipid accumulation and exerted little or no effect on PPAR-responsive enzymes and peroxisome proliferation*" (Moya-Camarena *et al.*, 1999). The data presented, the most definitive being histological evaluation of peroxisomes, supports this conclusion. Species differences between mouse and rat may explain the discrepancy in results. Moya-Camarena *et al.* (1999) speculated that these differences might be explained by differences in metabolism, resulting in increased sensitivity (sometimes resulting in false positives) of the mouse model to peroxisome proliferators. (Table 12)

7.4.1.3. Hamsters

de Decker *et al.* (1999) investigated the effect of a CLA mixture[160], a purified CLA containing *c*9,*t*11-isomer[161] or a purified CLA containing *t*10,*c*12-isomer[162] on organ weights[163], plasma total cholesterol and triacylglycerol, lipoproteins[164], liver lipids[165],

[160] CLA mixture was obtained from safflower oil (70 g linoleic acid/100g oil) and contained equal concentration of *c*9,*t*11-CLA (2.43 g/100 g total fatty acids) and *t*10,*c*12-CLA (2.44 g/100g total fatty acids).
[161] Concentration of *c*9,*t*11-CLA and *t*10,*c*12-CLA isomers were 4.53 and 0.32 g/100 g total fatty acids, respectively. The *c*9,*t*11-CLA isomer was incorporated into triacylglycerol (TAG).
[162] Concentration of *c*9,*t*11-CLA and *t*10,*c*12-CLA isomers were 0.48 and 3.95 g/100 g total fatty acids, respectively. The *t*10,*c*12-CLA was incorporated into triacylglycerol (TAG).
[163] Organ weights included liver, kidney and fat pads.
[164] Lipoproteins included VLDL-cholesterol, VLDL-triacylglycerol, LDL-cholesterol and HDL-cholesterol.

peroxisomal proliferation indices[166] and hepatic histopathology in male F_1B hybrid hamsters (Table 12). Fat, starch and cholesterol content of all diets were maintained at 131 (30% energy), 557 and 0.1 g/kg, respectively. Hamsters were fed the basal diet without CLA for two weeks prior to initiating treatment. Then, hamsters were fed either the basal diet (control), 6 g/kg of either CLA mixture (3 g *c*9,*t*11- and 3 g *t*10,*c*12- isomers *per* kg *per* day,), *c*9,*t*11-CLA (5.6 g *c*9,*t*11- and 0.4 g *t*10,*c*12- isomers *per* kg *per* day) or *c*10,*t*12-CLA (0.6 g *c*9,*t*11- and 4.9 g *t*10,*c*12- isomers *per* kg *per* day) for 8 weeks (*n*=32 *per* group). Body weights were recorded once weekly. Food intake was determined on weeks three and eight. Blood samples were taken after a 16-hour fast from the retro-orbital sinus. After eight weeks of treatment, liver, epididymal fat pads and kidneys were removed and weighed.

Weight gain, plasma total cholesterol and hepatic lipids were unaffected by any of the three CLA treatments in hamsters. Food intake was unaffected in *c*9,*t*11-CLA fed hamsters, and reduced 6% in hamsters fed the *t*10,*c*12-CLA or CLA mixture diets. Liver weights were increased 8%, 25% and 26% in *c*9,*t*11-CLA, *t*10,*c*12-CLA and CLA mixture diets, respectively. Weights of fat pad and kidney were unaffected in *c*9,*t*11-CLA treated hamsters. In *t*10,*c*12-CLA fed hamsters, fat pad and kidney weights were reduced 16% and increased 9%, respectively. Hamsters fed the CLA mixture also resulted in reduced fat pad weight (9%) and increased kidney weight (6%). Plasma total cholesterol and triacylglycerol were unaffected by *c*9,*t*11-CLA. Total cholesterol was reduced 13% in hamsters fed *t*10,*c*12-CLA or CLA mixture diets after four weeks of treatment, but was unaffected after eight weeks of treatment. Plasma triacylglycerol was increased 53% and 52% in hamsters fed *t*10,*c*12-CLA for four and eight weeks, respectively. Hamsters fed the CLA mixture diet also resulted in increased plasma triacylglycerol 38% and 62% after four and eight weeks of treatment, respectively. Plasma lipoproteins were unaffected in hamsters treated with *c*9,*t*11-CLA. Hamsters fed *t*10,*c*12-CLA and CLA mixture diets resulted in increased plasma VLDL 50-61% and 67-80%, respectively; whereas, plasma LDL-/HDL-cholesterol were reduced 18%/11% and 21%/8%, respectively. Peroxisome proliferation indices[167] were unaffected by any of the CLA treatments in hamsters. Liver histopathology revealed that the number of hepatocyte nuclei *per* area was reduced 13%, 24% and 18% in *c*9,*t*11-CLA, *t*10,*c*12-CLA and

[165] Liver lipids included total cholesterol and triacylglycerol.
[166] Peroxisomal proliferation indices included palmitoyl CoA oxidase activity, carnitine acetyl transferase activity in liver homogenates.
[167] Liver palmitoyl CoA oxidase and carnitine acetyl transferase activities.

CLA mixture treated hamsters, respectively. The histopathology indicates that all three CLA diets produced liver hypertrophy in hamsters. However, histopathology did not reveal any other differences between CLA-treated and control groups (de Deckere *et al.*, 1999).

Unlike the response in mice (Belury *et al.*, 1997), the results by de Decker *et al.* (1999) indicate that CLA does not affect peroxisome proliferation indices in hamsters. The dose of CLA used in this study was high (6 g/kg/day), thus, the lack of response would not seem to be due to inappropriate dose selection. This study demonstrates that the two main CLA isomers have different capacities to elicit a biological response. For instance, the *t*10,*c*12-CLA isomer produced most of the biological effects observed in hamsters. The only biological effect that *c*9,*t*11-CLA produced was increased liver weights and hepatic hypertrophy, no other biological effect was observed. The *t*10,*c*12-CLA isomer also increased liver weights and hepatic hypertrophy in hamsters, which were increased to a greater extent than what was observed for *c*9,*t*11-CLA treatment. The increase in liver weights in hamsters is consistent with other rodent studies (see section 7.1, page 31). Nevertheless, these data suggest that CLA does not induce peroxisomal proliferation in hamsters, and that species differences may exist between mice and hamsters. Because these two studies measured different peroxisomal proliferation indices, an alternative explanation is that the indirect peroxisomal proliferation indices measured by Belury *et al.* (1997) and de Decker *et al.* (1999) are not correlated. (Table 12)

7.4.2. Mechanism – peroxisomal proliferation

Xenobiotic induced peroxisome proliferation[168] is mediated by activation of PPAR-α (Lee *et al.*, 1995; Peters *et al.*, 1997). CLA has been shown to activate PPAR-α in *in vitro* trans-activation assays (Moya-Camarena *et al.*, 1999; Clement *et al.*, 2002). Moya-Camarena *et al.* (1999) reported that the *t*9,*c*11- isomer was the most effective isomer at inducing PPAR-α *in vitro*. Clement *et al.* (2002) reported that both *t*10,*c*12- and *c*9,*t*11- isomers activated PPAR-α *in vitro*. Activation of PPAR-α by CLA has not been demonstrated *in vivo*, although Meadus (2003) and Clement *et al.* (2002) reported that PPAR-α was not activated *in vivo* in CLA treated pigs and rats, respectively.

[168] Endogenous substances (*e.g.*, dehydroepiandrosterone 3 *beta*-sulphate) are also known to induce peroxisome proliferation (Ram and Waxman, 1994; Zhou and Waxman, 1998).

7.4.3. Toxicological significance – peroxisomal proliferation

Following critical evaluation of the available literature on species differences of peroxisomal proliferation, Roberts (1999) concluded that *"...humans differ from rodents in their response to PPs and the weight of evidence supports the supposition that PPs do not pose a carcinogenic risk to humans."*

7.5. Milk fat

The effect of CLA on milk fat, fatty acids and the growing neonate has been investigated in mice, rats and cows (Loor and Herbein 1998; Yang *et al.* 2002; Loor *et al.* 2003). These studies are toxicologically relevant because specific fatty acids (*i.e.*, arachidonic acid and docosohexanoic acid) play an important role in the developing neonate (*e.g.*, immune function, eye sight, and neurological development) (Innis, 2003; Uauy *et al.*, 2003). Therefore, the experimental studies in which the effect of CLA on milk fat are critically evaluated.

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7.5.1. Critical evaluation of experimental studies - milk fat

7.5.1.1. Mice

Loor *et al.* (2003) investigated the effect of purified CLA isomers (*c*9,*t*11- and *t*10,*c*12-CLA) on dam food intake, fat concentration in breast milk, breast milk fatty acid composition pup weight gain in mice. Beginning on day 4 of lactation, female mice (CD-1; *n*=5 *per* group) were fed either a basal diet or the basal diet supplmented with *c*9,*t*11- or *t*10,*c*12-CLA (dose was ~2.7 g/kg body weight). Treatment continued throughout lactation (*i.e.*, day 14 postpartum). Average food intake throughout the treatment period of dams for control, *c*9,*t*11- and *t*10,*c*12-CLA groups were 18.26, 18.45 and 15.71 g/day, respectively.

At the end of the treatment period, feeding dams *c*9,*t*11-CLA had no effect on dam body weight, food intake, dam carcass protein and fat weights, pup body weight, pup liver weight and pup carcass protein and fat weights. Food intake was reduced 16% (day 13 postpartum) to 25% (day 6 postpartum) in mice treated with 2.7 g *t*10,*c*12-CLA/kg body weight. Body weight was reduced 8% in dams fed *t*10,*c*12-CLA isomer; while carcass fat

weight[169] was reduced 40%. In pups of dams fed *t*10,*c*12-CLA isomer, body, liver and carcass fat weights were reduced 25%, 32% and 60%, respectively. Concentration of fat in dams milk was unaffected by *c*9,*t*11-CLA, and reduced 25% in dams fed *t*10,*c*12-CLA. Table 13 presents the effects (relative to control) of *c*9,*t*11- and *t*10,*c*12-CLA isomers on mouse milk fatty acid composition. Saturated fatty acids (SFA) of medium chain length (*i.e.*, 12 to 16 carbons) were reduced 10% and 67% by *c*9,*t*11- and *t*10,*c*12-CLA isomers, respectively. Longer chain SFA, monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) were unaffected by *c*9,*t*11-CLA. The effects of *t*10,*c*12-CLA on long chain MUFAs and PUFAs were variable (*i.e.*, increased 18:0, 18:1, 18:2, 20:6 and reduced 20:4).

Table 13. Effect of *c*9,*t*11-CLA and *t*10,*c*12-CLA on mouse milk fatty acids (Loor *et al.* 1998)

		Treatment Group	
Fatty Acid	Chain Length	*c*9,*t*11-CLA	*t*10,*c*12-CLA
Lauric, Myristic & Palmitic acids	12:0, 14:0, 16:0	↓ 10%	↓ 67%
Palmitoleic acid	16:1	NC	NC
Stearic acid	18:0	NC	↑ 20%
Oleic acid	18:1	NC	↑ 13%
Linoleic acid	18:2*n*6	NC	↑ 2%
α-Linolenic acid	18:3*n*3	NC	NC
Dihomo-γ-linolenic acid	20:3*n*6	NC	NC
Arachidonic acid	20:4*n*6	NC	↓ 43%
Eicosapentanoic acid	20:5*n*3	NC	NC
Docosahexanoic acid	22:6*n*3	NC	↑ 57%

Loor *et al.* (1998) postulated that the observed effects of *t*10,*c*12-CLA on maternal milk fat to be due to reduced mammary δ-9-desaturase activity; however, it is unclear whether any of the observed biological effects observed in this study were due to *t*10,*c*12-CLA isomer, or were due to the reduced food intake of the lactating dams treated with this isomer. It is plausible that the decreased body weight (dam and pups), reduced milk fat and reduced carcass fat was due to reduced intake of dietary fat and other nutrients. Thus, the reduced food intake was a confounding factor in this study, the influence of which was not adequately eliminated (usually by complex correlational analysis). The results of this study are also limited because (1) a dose-response relationship between individual CLA isomers and milk fatty acids were not investigated and (2) individual CLA isomers were administered, rather than a 50:50 ratio of the two isomers (*i.e.*, Tonalin®). Because of these limitations and

[169] Relative and absolute weights were reduced.

confounding factors, it is equivocal whether 2.7 g/day of *t*10,*c*12-CLA had any biological effect. It should be remembered that 0.5 g CLA/kg body weight fed to lactating dams actually enhanced rat pup growth (Chin *et al.* 1994; Poulos *et al.* 2001)[170].

7.5.1.2. Rats

Yang *et al.* (2002) investigated the effect of CLA[171] on fatty acid (especially *n*6 and *n*3) composition of dams milk and pup liver. Female Sprague-Dawley rats (4-5 *per* group) were fed either a basal diet (*i.e.*, control) or the basal diet supplemented with CLA (2% of diet or 2000 mg/kg body weight[172]).[173] Fatty acid content, except CLA, was similar between the control and CLA supplemented diets (Table 14). After 7 and 14 days of treatment, milk from the stomachs of five pups from each lactating dam were sampled to assesss milk intake and fatty acid content. Pup livers were also sampled for fatty acid (in phospholipid) analysis.

Table 14. Total fatty acid content of control and CLA supplemented diets (Yang *et al.*, 2002)

		Treatment Group g/kg diet (% total fatty acids)	
Total Fatty Acid	Chain Length	Control	CLA
Saturated fatty acids	16:0 – 22:0	8.6 (9.1%)	10.5 (9.1%)
Monounsaturated fatty acids	16:1n7 – 22:1n9	58.4 (62%)	62.2 (54.2%)
trans-Fatty acids	NR	0.6 (0.6%)	6.9 (6.0%)
CLA	18:2	<0.1 (<0.1%)	14.6 (12.7%)

NR = not reported.

Conjugated linoleic acid had no effect on pup body weights, liver weights or amount of stomach milk after 7 or 14 days of treatment. The effect of CLA on fatty acids in dam breast milk and pup liver phospholipids is presented in Table 15. Of the 17 fatty acids analyzed, six were significantly different from the control group. Lauric acid (12:0; milk only), γ-docosapentanoic acid (22:5*n*6; pup liver only) and α-linolenic acid (18:3*n*3; milk only) were reduced 17% - 67%; however, the reductions occured on only one of the two time points measured and in either breast milk or pup liver, but not both. Gadoleic acid (20:1*n*9) was increased 67% in breast milk and unaffected in pup liver. Myristic acid in breast milk was reduced 35% and 38% after 7 and 14 days of treatment, and unaffected in pup liver. Oleic

[170] Chin *et al.* 1994 did not specify the isomer content of CLA. Poulos *et al.* 2001 administered CLA mixture that is equivalent to Tonalin® (*i.e.*, 80% CLA of 50:50 *c*9,*t*11 and *t*10,*c*12).
[171] Specific isomer content of CLA administered was not reported.
[172] PAFA conversation factor of 1000 was used to convert % of diet to mg/kg (PAFA, 1993).
[173] The specific day of lactation on which CLA feeding started was not reported.

acid was reduced in breast milk (only after 7 days of treatment) and pup liver (after 7 and 14 days of treatment) 9% - 14%. Total lipid content of breast milk was reduced 33% by CLA treatment; however, total lipid content of pup liver was unaffected.

Table 15. Effect of CLA on breast milk (dam) and pup liver fatty acids (Yang *et al.*, 2002)

		Breast Milk Fatty Acids		Pup Liver Fatty Acids*	
Fatty Acid	Chain Length	7 Days	14 Days	7 Days	14 Days
Lauric	12:0	↓ 23%†	NC	NC	NC
Myristic	14:0	↓ 35%	↓ 38%	NC	NC
Palmitic	16:0	NC	NC	NC	NC
Stearic acid	18:0	NC	NC	NC	NC
Oleic acid	18:1*n*9	↓ 9%	NC	↓ 13%	↓ 14%
Gadoleic acid	20:1*n*9	NC	↑ 67%	NC	NC
Linoleic acid	18:2*n*6	NC	NC	NC	NC
γ-Linolenic acid	18:3*n*6	NC	NC	NC	NC
Eicosadienoic acid	20:2*n*6	NC	NC	NC	NC
Dihomo-γ-linolenic acid	20:3*n*6	NC	NC	NC	NC
Arachidonic acid	20:4*n*6	NC	NC	NC	NC
Docosatetraenoic acid	22:4*n*6	NC	NC	NC	NC
γ-Docosapentanoic acid	22:5*n*6	NC	NC	↓ 33%	NC
α-Linolenic acid	18:3*n*3	↓ 17%	NC	NC	NC
Eicosapentanoic acid	20:5*n*3	NC	NC	NC	NC
Docosapentanoic acid	22:5*n*3	NC	NC	NC	NC
Docosahexanoic acid	22:6*n*3	NC	NC	NC	NC
Total Lipids	n/a	↓ 33%	NC	NC	NC

* In phospholipids; † = Percent (%) relative to control; NC = not changed; n/a = not applicable.

These data indicate that feeding lactating dams a diet supplemented with a large dose of CLA does not affect pup body weight, liver weight or intake of milk. Only a few fatty acids in breast milk were affected by CLA treatment, all of which (except for oleic acid and γ-docosapentanoic acid[174]) were unaffected in pup liver. Thus, changes in breast milk fatty acid are not a good indicator of changes in pup liver fatty acid content, and that pup liver fatty acids are not as readily affected by CLA treatment in lactating dams. The lack of CLA's effect on pup rat liver fatty acids was probably due to exposure to lower dose of CLA than the lactating dams; because the CLA content (% of total fatty acids) pup liver was 10-fold lower than CLA content of breast milk (data not shown). The major limitations of this study are the lack of multiple doses administered to lactating dams and no developmental assessment (skeletal or visceral). (Yang *et al.*, 2002)

[174] γ-Docosapentanoic acid was affected after 7 days of treatment; whereas it was unaffected after 14 days of treatment.

7.5.1.3. Cows

Loor and Herbein (1998) administered, *via* abomasal[175] infusion, either 200 g of linoleic acid (LA) or a mixture containing linoleic acid (100 g) plus CLA[176] (100 g) (LCLA) for 24 hours to Holstein cows[177]. Blood and milk were collected every 12 hours for a total of 72 hours. Blood total fat and individual fatty acids (bound and free) were unaffected by LCLA treatment compared to LA treatment. Total free fatty acid in blood was increased 32% in cows infused with LCLA treatment. Individual free fatty acids were unaffected by LCLA treatment, except for free stearic acid, which was slightly increased (6%). Milk production was also unaffected by LCLA treatment; whereas milk composition of fat, protein and non-fat-solids were reduced[178] 19%, 5% and 3%, respectively, in cows administered LCLA. Yield of milk protein and non-fat-solids were unaffected by LCLA treatment, whereas milk fat was reduced 26%. The reduced amount of fat in milk was accompanied by a reduction (25%) in total fatty acid concentration in milk. Caproic acid was reduced 14%. The investigators reported that the reduction in total fatty acid yield was due to "*lower overall percentages and yield of palmitic and caproic acids, coupled with lower percentages...and yield of lauric and myristic acids*", and further reported that "*...CLA appeared to be a potent inhibitor of de novo fatty acid synthesis and desaturation in the mammary gland.*"

This study has limitations that include the short CLA infusion period (*i.e.*, 24 hours) and the CLA used was not representative of Tonalin® TG 80 (*i.e.*, contained primarily *c*9,*t*11-isomer rather than approximately equal amounts of *c*9,*t*11- and *t*10,*c*11-CLA isomers). Other CLA isomers were not analyzed, thus, the CLA administered to cows was not well characterized. Important long-chain polunsaturated fatty acids (c20), *e.g.*, arachidonic and docosohexanoic acids were not measured. Lastly, effect on calves reared on cows milk fed CLA was not investigated.

Chouinard *et al.* (1999) also investigated the effect of CLA[179] on milk fat production in Holstein cows[180] (between 215 and 301 days postpartum). CLA (0, 31.3, 57.7 and 90

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[175] Relating to the abomasum (the fourth compartment of the stomach of ruminants.
[176] CLA contained 35% *c*9,*t*11- and 15% *t*10,*c*12- isoforms. Other CLA isoforms was not reported.
[177] Cows were between 168 and 230 days postpartum.
[178] Use of relative terms (*e.g.*, increase, decrease, reduce) indicates statistical significance from either control or baseline throughout this document.
[179] The supplement administered contained 61.2% CLA. Specific CLA isomers included *c*9,*t*11- (14.5%); *c*10,*t*12- (21.1%); *c*11,*t*13- (10.6%); *c*8,*t*10- (9.3%). Other fatty acid constituents included oleic acid (20.8%), palmitic acid (6.4%) and stearic acid (2.9%).

g/day) was administered by abomasal infusion for five days. Milk was collected every twelve hours and analyzed for milk yield, total fat, individual fatty acids and protein. After the five-day treatment period, milk yield was unaffected in cows treated with 31.3 or 57.7 g/day; but was slightly reduced (15%) at the highest dose of CLA (90 g/day). The concentration of milk fat was reduced ~50% in cows treated with 31.3 g/day CL, and remained at this low level at the two higher doses. Milk protein was unaffected by CLA treatment. Dose-dependent reductions in short- and medium-chain fatty acids were observed; maximum reductions (35%-50%) occurred at the highest CLA dose (Table 16). Longer-chain fatty acids (*i.e.*, heptadecanoic, stearic, linoleic and linolenic acids) were increased (17%, 57%, 48% and 32%, respectively). The investigators also demonstrated that between 10.2%-26% of infused CLA was transferred to the milk fat.

Table 16. Effect of varying doses of CLA on cow milk fatty acids (Chouinard *et al.* 1999)

		Dose (g/day)		
Fatty Acid	Chain Length	31.3	57.7	90
Butyric acid	4:0	↓ 17%	↓ 18%	↓ 35%
Caproic acid	6:0	↓ 37%	↓ 43%	↓ 50%
Caprylic acid	8:0	↓ 44%	↓ 51%	↓ 53%
Capric acid	10:0	↓ 42%	↓ 46%	↓ 48%
Lauric acid	12:0	↓ 25%	↓ 25%	↓ 22%
Myristic acid	14:0	↓ 8%	↓ 13%	↓ 08%
Myristoleic acid	14:1	↓ 14%	↓ 41%	↓ 46%
Pentadecanoic acid	15:0	NC	NC	NC
Palmitic acid	16:0	NC	NC	NC
Palmitoleic acid	16:1	NC	NC	NC
Heptadecanoic acid	17:0	↑ 17%	↑ 24%	↑ 17%
Stearic acid	18:0	↑ 29%	↑ 61%	↑ 57%
Oleic acid	18:1	NC	NC	NC
Linoleic acid	18:2	↑ 35%	↑ 44%	↑ 48%
Linolenic acid	18:3	↑ 32%	↑ 39%	↑ 32%

The reduction in milk fat observed in this study by Chouinard *et al.* (1999) is consistent with other studies conducted in cows (Loor and Herbein 1998; Chouinard *et al.*, 1999). The main limitation of this study is that it does not report the consequence, if any, of reduced milk fat to calves. Thus, the toxicological significance in cows is uncertain.

[180] Body weight of the cows was not mentioned.

7.5.2. Mechanism – milk fat

The mechanism by which CLA reduces milk fat is not well understood. Loor *et al.* (2003) and Baumgard *et al.* (2000) administered purified CLA isomers (*c*9,*t*11- and *c*10,*t*12-CLA) to mice and cows, respectively, and indicated that it was the *c*10,*t*12-CLA isomer responsible for the reduction in milk fat. Baumgard *et al.* (2000) also reported that decreases in short- to medium chained fatty acids (*i.e.*, c4-c16) accounts (*i.e.*, 78%) for the reduction in total milk fat content in cows. Possible mechanisms that might be responsible for the reduction in milk fat include (1) reduced *de novo* fatty acid synthesis because mammary epithelial cells produce short- to medium-chain fatty acids, (2) increased lipolysis and fatty acid oxidation, (3) reduced fatty acid uptake into tissues and (4) reduced desaturation of long-chain fatty acids by δ-9-desaturase (Chouinard *et al.* 1999; Baumgard *et al.*, 2000). This latter mechanism is plausible because pig, mouse and human δ-9-desaturase and stearoyl-CoA-desaturase[181] activities are reduced by CLA treatment *in vitro* (Ntambi *et al.*, 1999; Choi *et al.*, 2000; Park *et al.*, 2000; Choi *et al.*, 2001; Smith *et al.*, 2002). However, other mechanisms of fatty acid pathways (*i.e.*, esterification, packaging and secretion) have not been investigated.

7.5.3. Toxicological significance - milk fat

Experimental studies by Loor and Herbein (1998), Yang *et al.* (2002) and Loor *et al.* (2003) indicate that CLA intake by lactating dams can alter certain fatty acids of the breast milk. For instance, data from all three species indicate that short-chain saturated fatty acids are either unaffected or reduced by CLA treatment. The effect of CLA on breast milk long-chain polyunsaturated fatty acids are not consistently reported between the three studies. Arachidonic and docosohexanoic acid are important mediators of neonatal development, of which, Loor *et al.* (2003) reports breast milk content of these polyunsaturated fatty acids to be reduced in mice whereas Yang *et al.* (2002) reports it to be unaffected in rats. However, data from both studies indicate that the ratio of *n*6:*n*3 fatty acids in breast milk is unaffected by CLA treatment of lactating dams. Thus, CLA does not appear to affect *n*6:*n*3 fatty acids in a manner that is considered to elicit an adverse effect in experimental animals. This conclusion is supported by the lack of changes in pup body weight and liver weight reported in the

[181] These enzymes catalyze the metabolism of CLA isomers.

studies by Yang *et al.* (2002) and Loor *et al.* (2003), as well as, studies by Chin *et al.* (1994) and Bee (2000) in which it was demonstrated that CLA actually enhances growth of rat pups and piglets. The enhancement of rat pup and piglet growth may be in part due to ability of CLA to increase incorporation of dietary docosohexanoic acid into tissue (Harris *et al.*, 2001). A search of the literature did not reveal any untoward effect(s) to experimental neonates (*i.e.*, animals) fed breast milk with a lower yield of fat. Based on these data, the effect of CLA on milk fat production in animals is of no apparent toxicological significance. Section 8.4, page 87, describes the effect of CLA on human breast milk fat.

7.6. Summary of other studies

CLA has been demonstrated to increase hepatic lipid accumulation, increase circulating insulin, increase aortic fat deposition, stimulate peroxisomal proliferation and reduce milk fat. Many of the effects observed in mice appear to species specific. For instance, mice appear to be the most sensitive species to CLA-induced hepatic lipid accumulation. In contrast to mice, only very high doses of CLA increase liver weight, but not hepatic vacuolization, in rats (O'Hagan and Menzel, 2003). Increased circulating insulin is also commonly seen in mice treated with CLA; however, insulin is much less affected in rats, and unaffected in pigs. No pathological or function effect has been demonstrated to be associated with induced hepatic lipid accumulation and increased circulating insulin in mice.

Fatty streaks, increases in peroxisomal proliferation indices and reduction in milk fat also do not appear to be of toxicological significance. The presence of fatty streaks has been demonstrated in only one study in which mice were fed CLA. Other mouse studies actually demonstrated an anti-aortic fat deposition effect in mice fed CLA. Increases in peroxisomal proliferation appear to be a species-specific phenomenon that occurs in mice, but not other species. There are questions about the reliability of indirect indices of peroxisomal proliferation. Reduced breast milk fat has been demonstrated in mice, rats and cows fed CLA. In rats, CLA does not alter levels of arachidonic or docosohexanoic acids in breast milk. CLA does not appear to adversely affect pup growth, but rather enhances neonatal growth in rats and/or pigs.

Increased hepatic lipid accumulation, increased circulating insulin concentration, fatty streaks, increase peroxisomal proliferation indices and reduce milk fat in animals do not appear to be toxicological significant.

8. Observations in humans

Numerous clinical and non-clinical studies involving CLA have been published in the biomedical literature in recent years (Ferreira *et al.*, 1997; Lowery *et al.*, 1998; Atkinson, 1999; Beuker *et al.*, 1999; Von Loeffelholz *et al.*, 1999; Basu *et al.*, 2000; Basu *et al.*, 2000; Berven *et al.*, 2000; Blankson *et al.*, 2000; Zambell *et al.*, 2000; Mougios *et al.*, 2001; Riserus *et al.*, 2001; Smedman and Vessby, 2001; Thom *et al.*, 2001; Zambell *et al.*, 2001; Kreider *et al.*, 2002). In some of these human studies, between 6-7.2 g *per* day of CLA was administered (Ferreira *et al.*, 1997; Lowery *et al.*, 1998; Beuker *et al.*, 1999; Von Loeffelholz *et al.*, 1999; Blankson *et al.*, 2000; Kreider *et al.*, 2002). The safety of CLA in humans was reported in a few studies (Berven *et al.*, 2000; Blankson *et al.*, 2000; Kreider *et al.* 2002). Nevertheless, studies deemed relevant to the safety of CLA in humans are critically evaluated in the following section, and summarized in Table 17, page 70.[182] The findings of CLA on clinical chemistry, including serum liver enzymes, and adverse events are emphasized. The effect of CLA on serum insulin concentration (section 8.2, page 77), endogenous levels of isoprostanes (8.3, page 83) and milk fat (8.4, page 87) are also discussed.

[182] Because the studies by Beuker *et al.* (1999) and Von Loeffelholz *et al.* (1999) are in a foreign language, these studies are not included in the following section.

Table 17. Summary of clinical studies in which CLA was administered orally to humans

Subjects (status/number)	CLA isomer(s) (ratio)	Dose(s) (g/day)	Duration (weeks)	Clinical Findings	Reference
			Healthy Adults		
Athletes (*n*=12)	CLA/isomer content were not reported	7.2	6	No significant difference in serum liver function tests (SGOT and SGPT enzymes) and serum glucose, lipids, BUN:creatinine ratio and LDH between CLA and control groups.	Lowery *et al.*, (1998)
Athletes (*n*=7)	54% CLA	7	24	Body weight, total body water, body fat and blood cholesterol, LDL cholesterol, HDL cholesterol and energy intake were unaffected. Blood chemistry and liver function tests not reported.	Von Loeffelholz *et al.*, (1999)
Athletes (*n*=23)	*t*10,*c*12 and *c*11,*t*13 (50:50)	6	4	Blood chemistry and serum liver function tests unaffected. No side effects, symptoms of impaired health reported.	Kreider *et al.* (2002)
Athletes (*n*=12)	NA	6	4	Body composition, BUN and creatinin were unaffected. Blood chemistry and liver function tests not reported.	Ferreira *et al.*, (1997)
Healthy Adults – M/F (*n*=25)	*c*9,*t*11 and *t*10,*c*12 (50:50)	4.2	12	Blood chemistry and liver function tests not reported. Urinary 8-iso-$PGF_{2\alpha}$ and 15-keto-dihydro-$PGF_{2\alpha}$ increased. Plasma MDA and serum α-tocopherol were unaffected. Serum γ-tocopherol (lipid corrected) was increased.	Basu *et al.* (2000)
Athletes (*n*=13)	NA	3	12	Reduced (15%) plasma cholesterol. Body weight and lean body mass were unaffected. Blood chemistry and liver function tests not reported.	Beuker *et al.*, (1999)**
Healthy Adults – F (*n*=17)	*t*10,*c*12 and *c*11,*t*13 (65% of 50:50)	3	9	Blood chemistry and liver function tests not reported. Plasma leptin transiently reduced.	Medina *et al.* (2000)
Healthy Adults – M/F (*n*=33)	*c*9,*t*11 and *t*10,*c*12 (50:50) *c*9,*t*11 and *t*10,*c*12 (80:20)	3	8	Serum liver function tests were not reported. Plasma triacylglycerol levels reduced. Plasma triacylglycerol in this group was not significantly different from the placebo group. Plasma cholesterol, non-esterified fatty acids (NEFA), glucose and insulin were unaffected. VLDL-cholesterol was reduced; however, VLDL-cholesterol was not significantly different from the placebo group. Other lipoproteins were unaffected. Incorporation of *c*9,*t*11-CLA into plasma lipids was increased. Supplementation with 50:50 CLA, incorporation of linolenic acid (18:3 *n*-3) was reduced. In subjects treated with 80:20 CLA, incorporation of eicosa-5Z,8Z,11Z,14Z,17Z-pentaenoic acid was reduced.	Noone *et al.* (2002)

Subjects (status/number)	CLA isomer(s) (ratio)	Dose(s) (g/day)	Duration (weeks)	Clinical Findings	Reference
				Lactating Women	
Healthy Lactating Women (*n*=9)	*c*9,*t*11 and *t*10,*c*12 (80%; 50:50)	1.5	0.7	Blood chemistry and liver function tests not reported. Plasma fatty acids were unaffected. Plasma *c*9,*t*11-CLA and *t*10,*c*12-CLA were increased. Milk fatty acids were unaffected. Milk *c*9,*t*11-CLA and *t*10,*c*12-CLA levels were increased. Total milk fat content was reduced. CLA treatment had no effect on infant milk consumption.	Masters *et al.* (2002)
				Overweight/Obese Adults	
Obese (*n*=60)	*c*9,*t*11 and *t*10,*c*12 (50:50)	1.7, 3.4, 5.1 or 6.8	12	Serum liver function tests unaffected. Blood lipids, potassium, creatinine and platelets reduced at 3.4 g CLA *per* day. Serum creatinine and bilirubin were reduced at 5.1 g CLA *per* day. CPK was reduced at 6.8 g *per* day. These effects were not dose-related. Twenty-six adverse events were reported to be gastrointestinal-related.	Blankson *et al.* (2000)
Obese – M (*n*=14)	*c*9,*t*11 and *t*10,*c*12 (50:50)	4.2	4	Blood chemistry and liver function tests not reported. Urinary 8-iso-$PGF_{2\alpha}$ and 15-keto-dihydro-$PGF_{2\alpha}$ levels increased. Urine 2,3-dinor-TXB_2 and serum α- and γ-tocopherol levels were unaffected. Two- and four-weeks after the cessation of CLA-treatment, urinary 8-iso-$PGF_{2\alpha}$ and 15-keto-dihydro-$PGF_{2\alpha}$ levels returned to baseline.	Basu *et al.* (2000)
Healthy Adults (*n*=38)	*c*9,*t*11 and *t*10,*c*12 (80% 50:50)	3.4	104	Blood chemistry and liver function tests unaffected. Body weight, BFM, LBM and BMI were reduced 10% or less. Plasma leptin, insulin and isoprostanes were unaffected.	Gaullier *et al.*, (2004); Cognis Corporation Nutrition and Health (2003)
Overweight Adults – M/F (*n*=27)	50:50 mixture (specific isomers were not reported)	1.8, 3.6	13	No treatment related adverse events. Blood chemistry and liver function tests not reported. Feelings of satiety and fullness increased, while hunger decreased. Energy intake and body weight regain were unaffected.	Kamphuis *et al.* (2003a)
Overweight Adults – M/F (*n*=27)	50:50 mixture (specific isomers were not reported)	1.8, 3.6	13	Serum liver function tests were not reported. Body weight regain unaffected. Body fat mass was reduced when expressed as a percentage, but was unaffected when expressed as absolute fat mass. Fat-free mass was increased. Plasma glucose, insulin, triglyceride, free fatty acid, glycerol and β-hydroxybutyrate were unaffected.	Kamphuis *et al.* (2003b)
Obese – M/F (*n*=60)	NR	3.4	12	Blood chemistry and serum liver function tests were unaffected. Clinically relevant adverse events were not detected.	Berven *et al.* (2000)

NA = not available; NR = not reported; **In German.

8.1. Safety Studies - blood chemistry, liver function and adverse events

The safety of CLA in humans has been investigated by Berven *et al.* (2000), Blankson *et al.* (2000) and Kreider *et al.* (2002). Specifically, the investigators studied whether CLA altered blood chemistry, affected liver function (serum liver enzymes) or increased the incidence of adverse events.

SECTION OVERVIEW

Animal Studies
6.1 – 6.7 Toxicity – *NOAEL 2.4/2.7 g/kg/d*
Other Biological Studies
7.1 Hepatic lipids – *no toxicological concern*
7.2 Insulin – *no toxicological concern*
7.3 Aortic fat deposition - *equivocal*
7.4 Peroxisomes – *species specific*
7.5 Milk fat – *no toxicological concern*
Human Studies
8.1 Safety
8.2 Insulin
8.3 Isoprostanes
8.4 Milk fat
8.5 Higher dose non-safety
8.6 Lower dose non-safety

8.1.1. Critical evaluation of human safety studies – Blood chemistry, liver function and adverse events

In a relatively large cohort, Berven *et al.* (2000) investigated the safety of CLA[183] in overweight or obese humans. Sixty subjects (male and female 18 years of age or older) were included in this study. All subjects had a body mass index (BMI) between 27.5 and 39.0 kg/m^2. The study design was a randomized, placebo controlled, double blind with two parallel groups. Treatment consisted of daily intake of capsules (six *per* day) containing either CLA[184] or placebo (olive oil). Two capsules were consumed prior to breakfast, lunch and dinner. The total daily dose of CLA was 3.4 grams *per* day. Total duration of treatment was twelve weeks. Complete demographic data on the participating subjects were collected prior to treatment. Safety parameters measured included: clinical examination (at baseline and 12 week), clinical chemistry[185] (at baseline and 12 week), vital signs (at baseline, 6 week and 12 week) and adverse events (throughout the treatment period) (see Table 17).

Of the 60 subjects that started the study, 55 completed the entire 12-week CLA treatment period. No differences in demographic data, medical history or concomitant medications were found between treated and control groups. Two subjects treated with CLA withdrew from the study because loss of interest (n=1) and to "*bad oral smell and bad smell of perspiration*" (n=1). Three subjects treated with placebo withdrew from the study because of lack of interest (n=1), cessation of treatment during holiday (n=1) and termination due to

[183] CLA was Tonalin™ obtained from Natural Lipids, Norway.
[184] Each capsule contained 750 mg safflower oil that contained a minimum of 75% CLA. CLA isomer composition was not characterized.
[185] Blood lipids (triglycerides, total cholesterol, LDL cholesterol, HDL cholesterol, lipoprotein (a)); hematology (hemoglobin, erythrocytes, white blood cells and platelets); liver (AST, ALT, bilirubin, *gamma*-glutamyl transferase); blood electrolytes (calcium, chloride, potassium, sodium); creatinine phosphokinase; glycated hemoglobin alc; lactate dehydrogenase; S-creatinine; S-ferretin; S-lipase.

gastrointestinal bleeding (n=1). The number of subjects that completed the study was 25 and 22 for CLA and placebo groups, respectively (Berven *et al.* 2000).

Blood chemistry and liver function: Compared to pre-treatment, blood bilirubin levels were reduced[186] (1%) in the CLA treated group, but this change was not significantly different from placebo group. Berven *et al.* (2000) determined that this change in bilirubin was not clinically significant. Other liver parameters (*i.e.*, AST, ALT and γ-GT[187]) were unchanged in both placebo and CLA groups. *S*-Creatinine was reduced 5% and 7% in the placebo and CLA treated groups, respectively, after 12 weeks of treatment compared to pre-treatment. The 2% difference in *S*-creatinine values between placebo and CLA groups was not statistically significant and were within clinical reference values, and hence, these changes were deemed clinically irrelevant. No other alterations in clinical chemistries were observed. Vital signs were normal in subjects treated with CLA.

Adverse events: Six subjects, three in each group, reported an adverse event that was described as "*mild to moderate*" in character. In the CLA group, two subjects experienced mild diarrhea that lasted for 30 and 77 days, which was untreated, and the conditions resolved on their own. The third subject experienced "*moderate bad smell*" and withdrew from the study voluntarily (see above). Berven *et al.* (2000) did not consider these effects to be clinical significant.

In the placebo group, one subject experienced mild diarrhea that lasted 16 days, which was untreated, and resolved on its own. Another subject experienced moderate gastritis/heartburn that lasted 76 days, which was untreated, and resolved on its own. The remaining subject in the placebo group experienced moderate gastritis/GI bleeding that lasted 5 days and withdrew from the study on the advice of a physician.

The findings from this study indicated that the oral administration of CLA at 3.4 g/day did not produce any adverse effects in humans (Berven *et al.*, 2000). There were very small reductions in blood bilirubin and *S*-creatinine levels in the CLA group after 12 weeks of

[186] Use of relative terms (*e.g.*, increase, decrease, reduce) indicates statistical significance from either control or baseline throughout this document.
[187] γ-Glutamyl transferase.

treatment, but these were determined to be clinically insignificant. The limitations of this study are the low number of subjects (22-25 *per* group) and the use of only one dose.

Blankson *et al.* (2000) (see Table 17) investigated the effect of CLA (Tonalin®, 50:50, Natural Lipids, Norway) on body fat mass in overweight and obese humans. Subjects in this study had a body mass index (BMI) between 25 and 35 kg/m^2 and were over 18 years of age. Subjects were screened and excluded based on (a) medical treatment for weight loss or other clinical conditions[188] and (b) pregnant or lactating women. The study design was a randomized, double blind, placebo-controlled with five parallel groups. Sixty subjects participated in the study and each randomly assigned into one of five groups (placebo, 1.7, 3.4, 5.1 or 6.8 g CLA[189] *per* day). Daily treatment consisted of six capsules (*i.e.*, two capsules prior to breakfast, lunch and dinner). Duration of treatment was 12 weeks. Each capsule contained 750 mg of oil. Placebo contained olive oil. Demographic data[190] was obtained from each. Each subject was evaluated clinically prior to treatment, then at six and twelve weeks of treatment. Clinical assessment consisted of body fat mass (BFM), lean body mass (LBM), body weights, blood pressure and heart rate. Blood samples were obtained prior to treatment and after 12 weeks of treatment and analyzed for hemoglobin, erythrocytes, white blood cells, platelets, serum creatinine, calcium, sodium, chloride, potassium, serum creatine phosphokinase, lactate dehydrogenase, alanine transaminase, aspartate transaminase, serum ferretin, γ-glutamyl transferase, bilirubin, glucosylated hemoglobin A_{1c}, serum lipase (activity), triglycerides, total cholesterol, LDL cholesterol, HDL cholesterol and lipoprotein (a). For adverse events, severity, frequency, action taken and outcome were also recorded. Body composition was measured by dual-energy X-ray absorptiometry. Statistical significance between means was determined using ANOVA, repeated measures ANOVA or ANCOVA. Post hoc testing was performed using either the Dunnett's test or Fisher's exact test (P value ≤ 0.05).

Demographic data in this study (see Table 17) did not differ between placebo and treatment groups. Of the 60 subjects that began treatment, 47 completed the study. Eight subjects withdrew during the first six weeks; whereas another five withdrew between six and

[188] Clinical conditions included diabetes, unstable or psychiatric illness.
[189] CLA (Tonalin™, Natural Lipids, Norway) consisted of 37.5% *c*9,*t*11 and 37.5% *t*10,*c*12 isomers (total CLA content was 75%).
[190] Demographic data included age, gender, height, weight, smoking and alcohol consumption.

twelve weeks of treatment. Of these 13 subjects, eight withdrew because of adverse events and five withdrew due to lack of interest. The number of subjects that withdrew from the study did not differ between placebo and treated groups. Compliance in taking the placebo CLA capsules was greater than or equal to 84%. After twelve weeks of treatment, body weights or BMI[191] were unaffected by CLA treatment. Significant differences in the change in BFM over the twelve-week treatment period between placebo and treated groups (i.e., 1.7, 3.4 and 6.8 g/day) were observed. Subjects treated with 3.4 g CLA *per* day had the greatest reduction (reduced by 1.7 kg) in BFM. LBM was increased[192] by 0.88 kg only in the 6.8 g CLA *per* day group. (Blankson *et al.* 2000)

Blood chemistry and liver function: Serum liver enzymes[193] were unaffected by CLA treatment in humans. In the placebo group, a significant increase in glucose was detected. In the three CLA treated groups, blood lipids were reduced. Blood potassium, creatinine and platelets were reduced in subjects treated with 3.4 g CLA. In the 5.1 g CLA group, serum creatinine and bilirubin were reduced; whereas in the 6.8 CLA group, creatine-phosphokinase was reduced. No other changes in blood chemistry were observed in treated *versus* placebo groups. Heart rate and blood pressure were unaffected by CLA treatment.

Adverse events: Thirty-six subjects were initially involved in a 12-week treatment period with CLA. The frequency of adverse events did not differ between the treatment groups. Of these 36 subjects, eight subjects withdrew due to adverse events that were determined, by the investigators, to be unrelated to treatment. In the remaining 26 subjects, adverse events were gastrointestinal-related. The number of adverse events in the placebo, 1.7, 3.4, 5.1 or 6.8 g CLA/day groups were 3, 5, 9, 8 and 11, respectively. Although Blankson *et al.* (2000) suggested that these gastrointestinal events could have been treatment related, the frequency of treatment related events did not differ between the five groups. This study by Blankson *et al.* (2000) was one of the few studies that used multiple doses of CLA, and a high dose of 6.8 grams *per* day.

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[191] Body mass index.
[192] 'Use of 'increase' or 'decrease' indicates statistical significance from either control or baseline throughout this document.
[193] Creatine phosphokinase, lactate dehydrogenase, alanine transaminase, aspartate transaminase, and *gamma*-glutamyl transferase.

Based on the data presented by Blankson *et al.* (2000), supplementation of the diet with CLA up to 6.8 grams *per* day did not result in any quantifiable biochemical adverse effect. Although adverse events that were gastrointestinal related were reported, the frequency of these events did not differ between the treatment groups.

Kreider *et al.* (2002) (see Table 17) investigated the effect on CLA[194] body composition, bone density, physical strength and selected hematological parameters[195] in athletes (gender not specified). Twenty-three athletes were carefully selected[196] and assessed[197] prior to administration of CLA. Then, each athlete was randomly assigned to either a placebo that contained olive oil (9 g/day) or CLA-treated (6 g/day)[198] group and treated for twenty-eight days. Placebo and CLA were administered in capsules that were ingested three times *per* day at mealtime (*i.e.*, morning, mid-day and evening). Nutritional intake was monitored throughout the study. After the twenty-eight day treatment, athletes did not exercise for 48 hours and fasted over night, and then blood was sampled for hematological parameters. Athletes completed a post-study questionnaire, which did not reveal any side effects, symptoms of impaired health or any other medical problem. CLA treatment had no effect on the hematological parameters measured, which included blood liver enzymes, indicating no adverse effect on the liver. The investigators reported moderate to large effects in urea nitrogen/creatinine and neutrophil/lymphocyte ratios in athletes whose diets were supplemented with CLA. Body mass was unaffected by CLA treatment. The investigators reported moderate to large changes in bone mass. CLA had no effect on strength performance.

Kreider *et al.* (2002) concluded that...

> "The serum and whole blood analyses performed in the present study revealed two main findings: (a) CLA supplementation appears to be relatively safe and does not promote clinically significant changes in general markers of health; and (b) although some potentially

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[194] Isomers in administered CLA were *t*10,*c*12- (22.6%), *c*11,*t*13- (23.6%), *c*9,*t*11- (17.6%), *t*8,*c*10- (16.6%), *t*9,*t*11- and *t*10,*t*12- (7.7%) and unidentified substances (11.9%). Total isomer content of administered CLA was 65%. CLA was obtained from Pharmanutrient, Inc. (Lake Bluff, IL).

[195] Hematological parameters that were measured, but data not shown, included total protein, albumin, globulin, glucose, electrolytes, liver enzymes, lipid profiles, total bilirubin, hemoglobin, hematocrit, red blood cells and white blood cells. Hematological data was presented for creatinine, blood urea nitrogen, urea nitrogen/creatinine ratio, creatine kinase, lactate dehydrogenase and neutrophil/lymphocyte ratio.

[196] Athletes were screened and selected based on training frequency/duration, type of exercises performed and no ingestion of creatine, CLA or *beta*-agonists during the prior two months.

[197] Blood sample was taken for hematological parameters, total body mass, total body water, total body composition and strength performance.

[198] Total fatty acid content was 9 grams *per* day. Fatty acids in the remaining 3 grams were not specified.

beneficial trends were observed in the ratio of urea nitrogen/creatinine (a general marker of catabolism) and neutrophil/lymphocytes (a general marker of immune stress), CLA supplementation did not significantly reduce markers of catabolism or immune stress."

The data presented support this conclusion. The limitations of this study are (1) number of subjects was small, (2) athletes rather than non-athletes were administered CLA and (3) the treatment period was short. The selection of athletes was chosen to assess the effect of CLA in this sub-group. Although the duration of treatment was short, previous studies (*e.g.*, Blankson *et al.* 2000; Basu *et al.* 2000) have administered a similar large dose of CLA to humans for a longer period.

8.1.2. Toxicological Significance - liver function, blood chemistries and other effects

These human safety studies indicate that CLA, up to 7.2 grams *per* day (highest dose tested) is tolerated, and does not produce any adverse effect on any of the parameters tested. Clinical chemistry data does not support that CLA produces any untoward effect in humans. The incidence of adverse events was low and mild in nature.

8.2. Effect of CLA on circulating insulin and leptin concentrations

SECTION OVERVIEW
Animal Studies
6.1 – 6.7 Toxicity – *NOAEL 2.4/2.7 g/kg/d*
Other Biological Studies
7.1 Hepatic lipids – *no toxicological concern*
7.2 Insulin – *no toxicological concern*
7.3 Aortic fat deposition - *equivocal*
7.4 Peroxisomes – *species specific*
7.5 Milk fat – *no toxicological concern*
Human Studies
8.1 Safety – *no adverse effect (AE) 0.1 g/kg/d*
8.2 Insulin
8.3 Isoprostanes
8.4 Milk fat
8.5 Higher dose non-safety
8.6 Lower dose non-safety

The effect of CLA on circulating concentration of insulin was investigated by Medina *et al.* (2000). Because increases in plasma insulin concentration have been implicated as a potential adverse effect in experimental animals, studies that investigated the effect of CLA on plasma insulin concentration in humans are critically evaluated in the following section.

8.2.1. Critical evaluation of human studies - insulin/leptin concentration

Medina *et al.* (2000) investigated the effect of CLA (Tonalin®, 65% CLA isomers, 50:50, Pharmanutrient, Illinois) on circulating levels of leptin, insulin, glucose and lactate concentrations in healthy women in a parallel, random, double blind study. Seventeen non-smoking women between the ages of 20 and 41 lived 24 hours *per* day, seven days a week in a metabolic suite for 94 days. The first 30 days was an acclimation period, followed by a 64-day treatment period. Women were randomly assigned to either the control group or the

CLA[199]-treated group. Control (containing sunflower oil[200]) and CLA (3 g/day) were administered in capsules. The diets of all subjects were equal in carbohydrate (55%), protein (15%) and fat (30%) content. Caloric intake was recorded and adjusted to maintain a consistent body weight. BMI, appetite and plasma concentrations of leptin, insulin, glucose and lactate were measured on treatment days 33, 49, 57 and 63. BMI was unaffected by CLA treatment compared to baseline or the control group. Mean plasma leptin concentration was reduced[201] 23 and 20% after 49-days of treatment compared to the control group and baseline, respectively. Although not statistically significant, mean plasma leptin concentration appeared to be reduced at the earlier time point (day 33), and appeared to be returning to normal levels (control and baseline) on treatment days 57 and 63. Mean plasma insulin, glucose and lactate concentrations were unaffected by CLA-treatment, as well as appetite.

Medina *et al.* (2000) (see Table 17) attempted to explain the reduced plasma leptin concentrations observed after 49 days of CLA treatment, by analyzing both leptin and insulin concentrations as percent change from baseline. There was a significant reduction in plasma leptin concentration (~18%) at the 33-day treatment period, but not at the other time points. Although plasma insulin concentration appeared to be higher in the CLA-treated group, these changes were not statistically significant. The investigators hypothesized that the "*apparent*" increase in plasma insulin concentration observed in the CLA-treated group mediated the return of reduced plasma leptin concentrations observed after 49 days of treatment. This hypothesis is based on reports by Saladin (1995) and Cusin (1995) who demonstrated that insulin increase plasma leptin concentration in humans. Alternatively, the investigators hypothesized that CLA may directly mediate the return of plasma leptin concentrations by activating the human peroxisome proliferator-activated receptor–γ (PPAR-γ), which has been demonstrated in rat and mouse PPAR-γ *in vitro* and *in vivo* (Houseknecht *et al.*, 1998; Kallen and Lazar, 1996; Zhang *et al.*, 1996).

Although the data from this study by Medina *et al.* (2000) suggests that CLA might reduce plasma leptin concentration in women, the effect on plasma insulin concentration was

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[199] A 65% CLA mixture of isomers that included *t*10,*c*12- (22.6%), *c*11,*t*13- (23.6%), *c*9,*t*11- (17.6%), *t*8,*c*10- (16.6%), *t*9,*t*11 & *t*10, *t*12- (7.7%) and 11.9% unidentified isomers. CLA was obtained from Pharmanutrient, Inc. (Lake Bluff, IL).

[200] Sunflower oil contained 72.6% linoleic acid and no detectable CLA.

[201] Use of relative terms (*e.g.*, increase, decrease, reduce) indicates statistical significance from either control or baseline throughout this document.

ambiguous. Further *in vivo* studies are needed to confirm the observed reduction in plasma leptin, because it was reduced in CLA-treated subjects at only one time point in this study. Additional studies are also needed demonstrating reduced plasma leptin concentration at multiple timpe points, its transient nature, and associated with significant changes in plasma insulin concentrations. This study included a small number of subjects, thus, future studies should increase this sample size considerably. Lastly, additional mechanistic studies testing the proposed hypotheses will also be beneficial.

Gaullier *et al.* (2004) (see Table 17) conducted a one-year randomized, double blinded, placebo-controlled clinical study in which healthy overweight subjects (ages 18-66 years) were assigned to a control (4.5 g of olive oil/day; n=45), CLA[202]-FFA[203] (3.4 g/day; n=44) or CLA-TG[204] (3.4 g/day; n=38) group. The duration of the study was twelve months. Blood samples were taken prior to CLA administration (*i.e.*, baseline), and then after 12-months of CLA-treatment. Body weights, body fat mass (BFM), lean body mass (LBM), body mass index (BMI), and clinical chemistry[205] was measured. After the completion of this one-year study, Cognis Corporation Nutrition and Health (2003) sponsored an additional twelve-months of treatment using the same subjects[206]. Results from the first and second twelve-month treatment periods are presented in Table 18, Table 19 and Table 20. Although statistical analysis indicates some significant differences on whole body parameters between (a) treated *versus* baseline (Table 18) and (b) treated and control (Table 19), the differences were quite small (less than 10%).

[202] CLA (Tonalin™, Natural Lipids, Hovdebygda, Norway) contained 80% of *c*9,*t*11 and *t*10,*c*12 (50:50).
[203] Free fatty acid.
[204] Triglyceride.
[205] Clinical chemistry parameters included alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), hemoglobin, bilirubin, chloride, creatine phosphokinase (CPK), erythrocytes, *gamma*-glutamyl transpeptidase (γ-GT), leukocytes, potassium, sodium, thyroid stimulating hormone (TSH), thrombocytes, thyroxin, glucohemoglobin, glucose, high density lipoprotein (HDL) cholesterol, insulin-like growth factor (IGF)-1, insulin, insulin c-peptide, low-density lipoprotein (LDL) cholesterol, leptin, lipoprotein (a), total cholesterol and triglycerides.
[206] The placebo group was not included during this second 12-month treatment period.

8.3. Serum isoprostanes

SECTION OVERVIEW

Animal Studies
6.1 – 6.7 Toxicity – *NOAEL 2.4/2.7 g/kg/d*
Other Biological Studies
7.1 Hepatic lipids – *no toxicological concern*
7.2 Insulin – *no toxicological concern*
7.3 Aortic fat deposition - *equivocal*
7.4 Peroxisomes – *species specific*
7.5 Milk fat – *no toxicological concern*
Human Studies
8.1 Safety – *no adverse effect (AE) 0.1 g/kg/d*
8.2 Insulin – *no toxicological concern*
8.3 Isoprostanes
8.4 Milk fat
8.5 Higher dose non-safety
8.6 Lower dose non-safety

Tissue injury due to oxidative mechanism(s) is of toxicological significance (Jaeschke *et al.*, 2002). Mechanism of oxidative tissue injury involves the generation of free radicals that cleave proteins, DNA and lipids, which results in further generation of free radicals. Free radicals are short-lived molecules that are difficult to measure *in vivo*. Indirect measures of free radical generation *in vivo* are often used as indicators of protein (oxidized amino acids), DNA (thymidine glycol, 8-hydroxy-2'-deoxyguanosine, 8-oxo-7,8-dihydro-2'-deoxyguanosine) and lipids (thiobarbituric acid reactive substances, exhaled pentane/ethane, isoprostanes) oxidation (Halliwell and Dizdaroglu, 1992; Simic, 1992; Reilly *et al.*, 1997; Anonymous, 2000; Lunec *et al.*, 2000; Griffiths, 2000; Cracowski *et al.*, 2000a; Cracowski *et al.*, 2000b; Morrow, 2000; Gopaul and Anggard, 2003; Mayne, 2003; Wood *et al.*, 2003).

In several studies, it has been reported that lipid peroxidation is induced in humans administered CLA (Basu *et al.*, 2000; Basu *et al.*, 2000; Basu and Vessby, 2001). Lipid peroxidation was measured by quantifying urinary isoprostane[211] levels. Endogenous isoprostanes is a commonly used marker for lipid peroxidation (Roberts and Morrow, 1994; Awad *et al.*, 1996; Morrow and Roberts, 1996; Morrow and Roberts, 1997; Reilly *et al.*, 1997; De Zwart *et al.*, 1999; Cracowski *et al.*, 2000a; Cracowski *et al.*, 2000b; Greco *et al.*, 2000; Morrow, 2000; Cracowski *et al.*, 2002; Pilacik *et al.*, 2002; Gopaul and Anggard, 2003). The effect of CLA on increasing endogenous isoprostane levels in humans is evaluated in the following section. Then, the potential toxicological significance of elevated endogenous isoprostane levels is discussed in section 8.3.2, page 86.

8.3.1. Critical evaluation of human studies – serum isoprostanes

Basu *et al.* (2000) (see Table 17) investigated the effect of CLA[212] (4.2 g/day) on endogenous isoprostane levels in normal healthy subjects (males and females, ages 23-63, *n*=25-28 *per* group). Blood and urine samples were collected during the initial two weeks of

[211] The term "*isoprostane*" refers to substances that are similar in structure to PGF_2 (an endogenous metabolite of arachidonic acid) that were originally identified as products of a non-enzymatic autoxidation mechanism in the 1960's (Morrow and II, 1997).
[212] CLA (Natural Ltd ASA, Oslo, Norway) contained 50:50 ratio of *c*9,*t*11- and *t*10,*c*12- isomers.

treatment with corn oil (*i.e.*, before treatment) and during the last week of treatment with corn oil (control group) or CLA (*i.e.*, after treatment). Urine samples were stored at –70° C[213]. Isoprostanes, 8-iso-$PGF_{2\alpha}$[214] and 15-keto-dihydro-$PGF_{2\alpha}$, were measured by a radioimmunoassay (RIA) method[215]. Urinary 8-iso-$PGF_{2\alpha}$ and 15-keto-dihydro-$PGF_{2\alpha}$, plasma MDA and serum α- and γ-tocopherol levels were unaffected. In the CLA-treated group, urinary 8-iso-$PGF_{2\alpha}$ measured in the morning and then 24 hours later were increased[216] 300 and 230%, respectively. Urinary 15-keto-dihydro-$PGF_{2\alpha}$, level was increased 86% after 12-weeks of treatment with CLA. Plasma MDA and serum α-tocopherol were unaffected by CLA treatment. Serum γ-tocopherol (lipid corrected) was increased 40% in volunteers treated with CLA for 12-weeks. Although plasma isoprostane concentrations were measured, the data was of limited value due problems in sample collection. Based on these data, Basu *et al.* (2000) concluded, "*...dietary supplementation of CLA induces both non-enzymatically and enzymatically catalysed lipid peroxidation in humans.*"

Basu *et al.* (2000) conducted a study in which the effect of CLA[217] (4.2 g *per* day) on urinary levels of 8-iso-$PGF_{2\alpha}$ and 15-keto-dihydro-$PGF_{2\alpha}$ in obese men[218] were investigated. Urinary 8-iso-$PGF_{2\alpha}$, 15-keto-dihydro-$PGF_{2\alpha}$ and 2,3-dinor-TXB_2, as well as serum α- and γ-tocopherol levels were measured before treatment, after 4 weeks of treatment, as well as at two and four weeks after cessation of treatment. In the control group, urinary and 2,3-dinor-TXB_2, as well as serum α- and γ-tocopherol levels, were unchanged over the four-week experimental period. In the CLA-treated group, urinary 8-iso-$PGF_{2\alpha}$ and 15-keto-dihydro-$PGF_{2\alpha}$ levels were increased 400 and 100%, respectively. Urine 2,3-dinor-TXB_2 and serum α- and γ-tocopherol levels were unaffected by CLA treatment. Two- and four-weeks after the cessation of CLA-treatment, urinary 8-iso-$PGF_{2\alpha}$ and 15-keto-dihydro-$PGF_{2\alpha}$ levels returned to baseline.

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[213] Freezing of urine samples at ultra-low temperatures is an important step; however, it is not as critical as quantifying isoprostanes in plasma or tissue because urine contains only trace amounts of lipid, thus negating the concern of autoxidation *ex-vivo* (Awad *et al.*, 1996).

[214] 8-iso-$PGF_{2\alpha}$. was measured in urine collected once in the morning followed by a second sample 24-hours later.

[215] RIA is highly dependent upon the specificity of the antibody used to detect the substance of interest. The antibody used to detect 8-iso-$PGF_{2\alpha}$ cross-reacted with other isoprostane substances that ranged between 0.01 and 9.8%. The antibody used to detect 15-keto-dihydro-$PGF_{2\alpha}$ cross-reacted with other isoprostane substances that ranged between <0.001 and 0.43%.

[216] Use of relative terms (*e.g.*, increase, decrease, reduce) indicates statistical significance from either control or baseline throughout this document.

[217] CLA (Natural Ltd ASA, Oslo, Norway) contained 50:50 ratio of *c*9,*t*11- and *t*10,*c*12- isomers.

[218] Obese men had a body mass index (BMI) between 31.4 and 32.2 kg/m^2, which were considered a cardiovascular disease high-risk group.

Basu *et al.* (2001) published an abstract in which the effects of specific CLA[219] isomers on urinary levels of 8-iso-$PGF_{2\alpha}$ and 15-keto-dihydro-$PGF_{2\alpha}$ and their regulation by vitamin E[220] (an antioxidant) and a cyclooxygenase (COX-2) inhibitor[221] were reported. Healthy men and women (*n*=60 *per* sex *per* group) were assigned to one of six treatment groups and then administered the COX-2 inhibitor (12 mg/day), vitamin E (200 mg/day) or neither (control) for six weeks. During the last four weeks of treatment, three groups were treated with a CLA (3 g/day) isomer mixture (*c*9,*t*10- and *t*10,*c*12-CLA) or with a single CLA (3 g/day) isomer (*t*10,*c*12-CLA). Treatment with a COX-2 inhibitor or vitamin E during the first two weeks of the study period did not alter urinary levels of 8-iso-$PGF_{2\alpha}$ and 15-keto-dihydro-$PGF_{2\alpha}$. Treatment with the CLA isomer mixture or with *t*10,*c*12-CLA increased urinary 8-iso-$PGF_{2\alpha}$ and 15-keto-dihydro-$PGF_{2\alpha}$ levels. The authors reported that these urinary constituents were increased to a greater level in subjects administered the single CLA isomer (*t*10*c*12-CLA) than subjects administered the CLA isomer mixture. Treatment with COX-2 inhibitor "*significantly reduced*"[222] urinary 15-keto-dihydro-$PGF_{2\alpha}$ levels in subjects administered the single CLA isomer, but not in the CLA isomer mixture group. The effect of vitamin E on urinary 15-keto-dihydro-$PGF_{2\alpha}$ levels was not reported. Urinary levels of 8-iso-$PGF_{2\alpha}$ were unaffected by COX-2 inhibitor or vitamin E. The authors concluded that CLA-induced changes in endogenous isoprostanes might partially be mediated by COX-2. Because limited information was provided in the Abstract for this experimental study, a critical evaluation of these findings cannot be performed.

Recently, a clinical double-blinded study was conducted by the Scandinavian Clinical Research AS and sponsored by Cognis Corp. Nutrition and Health (Syvertsen, 2003, unpublished report). Healthy subjects (ages 18-66 years) were assigned to a control (4.5 g of olive oil/day), CLA-FFA[223] (3.4 g/day) and CLA-TG[224] (3.4 g/day) group (treatment period was twelve months)[225]. Serum samples were obtained and analyzed for 8-iso-$PGF_{2\alpha}$ by gas chromatography/mass spectrometry (GC/MS) analysis using a chemical ionization detector.

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[219] Information regarding the identity of CLA used was not provided.
[220] *alpha*-Tocopherol.
[221] Trade name is Vioxx® (active ingredient is rofecoxib).
[222] Amount of reduction was not stated.
[223] Free fatty acid.
[224] Triglyceride.
[225] CLA (Tonalin™, Natural Lipids, Hovdebygda, Norway) contained 80% of *c*9,*t*11 and *t*10,*c*12 (50:50).

increase in isoprostanes *in vivo* by CLA treatment is unlikely to be of toxicological consequence.

8.4. Milk fat

Potential adverse effects to the neonate are of concern in safety assessments of food ingredients. A study by Masters *et al.* (2002) investigated the effect of CLA in human milk fat is critically evaluated.

SECTION OVERVIEW

Animal Studies
6.1 – 6.7 Toxicity – *NOAEL 2.4/2.7 g/kg/d*
Other Biological Studies
7.1 Hepatic lipids – *no toxicological concern*
7.2 Insulin – *no toxicological concern*
7.3 Aortic fat deposition - *equivocal*
7.4 Peroxisomes – *species specific*
7.5 Milk fat – *no toxicological concern*
Human Studies
8.1 Safety – *no adverse effect (AE) 0.1 g/kg/d*
8.2 Insulin – *no toxicological concern*
8.3 Isoprostanes - *equivocal*
8.4 Milk fat
8.5 Higher dose non-safety
8.6 Lower dose non-safety

8.4.1. Critical evaluation of human studies - milk fat

Masters *et al.* (2002) (see Table 17) investigated the effect of CLA[229] on the fat content in human breast milk. The study design was a randomized, double blind, crossover, placebo-controlled experiment with three periods. Healthy lactating women (n=9) were administered a placebo[230] for five days, then placebo was suspended seven days, and CLA administered for five days. Placebo and CLA were administered in capsules, two *per* day. Total daily dose of CLA was 1107 mg. Milk samples and non-fasting maternal blood were collected on the last day of each treatment period. Infant milk consumption was estimated by weighing each infant before and after feeding. Fatty acid content of milk samples was quantified by gas chromatography. Background rumenic acid (*c*9,*t*11-CLA) consumption, as well as other macronutrients[231], was assessed using a food frequency questionnaire (FFQ)[232].

Background consumption of CLA, rumenic acid (RA) and macronutrients were determined to be similar between CLA and placebo treatment periods, indicating that dietary intake of 1.5 g CLA *per* day does not reduce intake of other macronutrients in lactating women. Plasma fatty acids[233] were also unaffected by CLA treatment, whereas plasma *c*9,*t*11-CLA and *t*10,*c*12-CLA were increased during CLA treatment compared to placebo (data not

[229] Tonalin™ (Natural Lipids, Hovdebygda, Norway) contained ~80% CLA. Specific isomer content was *c*9,*t*11- (36.4%); *t*10,*c*12- (37.2%); *c*9,*c*11- (1.3%); *c*10,*t*12- (1.1%), plus, oleic acid (19.9%), palmitic acid (2.4%), linoleic acid (0.4%) and stearic acid (0.3%).

[230] Placebo was olive oil that contained oleic acid (75.2%), palmitic acid (10.9%), linoleic acid (6.7%), stearic acid (3.6%) and capric acid (2.5%).

[231] Macronutrients included energy, protein, carbohydrate, lipid, saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids.

[232] The investigators mentioned that FFQ method might "*somewhat*" underestimate an individual's intake of fatty acids. The underestimation by the FFQ method was thought to be insignificant because it has been shown that total CLA and rumenic acid intakes are "*significantly related to biochemically determined CLA intake.*"

[233] Caprylic acid (8:0), capric acid (10:0), lauric acid (12:0), myristic acid (14:0), pentadecanoic acid (15:0), palmitic acid (16:0), palmitoleic acid (16:1*n*-7), stearic acid (18:0), oleic acid (18:1*n*-9), linoleic acid (18:2*n*-6) and linolenic acid (18:3*n*-3).

shown). Milk fatty acids were also unaffected by CLA treatment, whereas *c*9,*t*11-CLA and *t*10,*c*12-CLA were increased during CLA treatment compared to placebo (Table 21). Total milk fat content was reduced[234] 25% during CLA treatment compared to placebo treatment. Importantly, CLA supplementation had no effect on infant milk consumption.

Table 21. Effect of CLA (1.5 g/day) on breast milk fatty acids (Masters *et al.* 2002)

Fatty Acid	CLA Treatment (μmol/g lipid)	Placebo Treatment (μmol/g lipid)	Change (%)	Statistical Significance
8:0	106.9 ± 94.4	14.6 ± 1.4	↑ (632%)	NS
10:0	83.1 ± 16.9	94.1 ± 20.9	↓ (12%)	NS
12:0	227.9 ± 27.0	227.9 ± 18.0	↔	NS
14:0	232.2 ± 23.2	239.3 ± 24.1	↓ (3%)	NS
15:0	14.3 ± 2.3	15.7 ± 2.1	↓ (9%)	NS
16:0	828.7 ± 63.2	803.7 ± 65.9	↑ (3%)	NS
16:1*n*-7	94.6 ± 11.9	87.2 ± 13.6	↑ (8.5%)	NS
18:0	225.5 ± 36.9	259.3 ± 32.7	↓ (13%)	NS
18:1*n*-9	1055.3 ± 72.3	1060.6 ± 61.3	↓ (0.5%)	NS
18:2*n*-6	468.6 ± 61.7	454.0 ± 42.1	↑ (3.2%)	NS
18:3*n*-3	41.7 ± 6.8	43.9 ± 6.8	↓ (5.0%)	NS
*c*9,*t*11-18:2	30.0 ± 2.1	15.3 ± 1.8	↑ (96.1%)	S
*t*10,*c*12-18:2	11.1 ± 2.1	ND		S

Values for CLA and Placebo are mean ± standard error of the mean (SEM); NS = not significant; S = significant.

This study by Masters *et al.* (2002) had several limitations, including 1) a small sample size (only 7 lactating women), 2) a short duration (10 non-consecutive days), 3) non-purified isoforms of CLA (*i.e.*, *c*9,*t*11-CLA and *t*10,*c*12-CLA), 4) the fatty acid composition of the placebo did not closely match the fatty acid composition of the CLA administered and 5) components that contributed to the reduction in percent milk fat were unexplained. Masters *et al.* (2002) concluded, "*consumption of currently available CLA supplements decreases milk fat content in women.*"

8.4.2. Toxicological significance – milk fat

Based on experimental studies in animals and dietary intake of CLA women, the reduced breast milk fat observed in lactating mothers is unlikely to result in an untoward effect in infants. Yang *et al.*, (2002) demonstrated in rats that despite a 33% reduction in milk

[234] Use of relative terms (*e.g.*, increase, decrease, reduce) indicates statistical significance from either control or baseline throughout this document.

fat, milk intake by pups, *n*6:*n*3 fatty acids (*e.g.*, arachidonic and docosohexanoic acid)[235], pup body weight and pup liver weight are unaffected by treatment of lactating dams with CLA.

Compared to other studies that investigated the amount of CLA in human breast milk, the values of *c*9,*t*11-18:2 and *t*10,*c*12-18:2 in females treated with 1.5 g/day reported by Masters *et al.* (2002) appear to be within the range of biological variation. For instance, Fogerty (1988) reported CLA (*i.e.*, *c*9,*t*11-18:2 and *t*10,*c*12-18:2) in normal human breast milk to be 40 µmol/g fat, which is approximately equal to the 41.1 µmol/g lipid (*i.e.*, 30 + 11.1) observed in women treated with 1.5 g CLA *per* day by Masters *et al.* (2002).

The influence of diet on the fat content of human breast milk is not surprising. Forgerty (1988) hypothesized that the 40 µmol CLA/g fat in breast milk of lactating women was due to greater use of butter or ghee in their foods[236]. Francois *et al.*, (1998) demonstrated that menhaden (20g bi-weekly for 10 weeks), herring (7g bi-weekly for 10 weeks), safflower (40g bi-weekly for 10 weeks), canola (40g bi-weekly for 10 weeks) and coconut (40g bi-weekly for 10 weeks) oils, as well as cocoa butter (40g bi-weekly for 10 weeks), alter the fatty acid composition of human breast milk. Francois *et al.*, (1998) reported a reduction in total saturated fatty acid (SFA), total monounsaturated fatty acid (MUFA) and/or total polyunsaturated acid (PUFA) in women that consumed herring oil (reduced SFA), safflower oil (reduced SFA and MUFA), canola oil (reduced MUFA) and cocoa butter (reduced PUFA). Francois *et al.*, (1998) implied that the changes to fatty acid composition (*i.e.*, SFA, MUFA and PUFA) were not biologically significant.

Animal and clinical studies have demonstrated a positive effect on infants of mothers fed CLA or long-chain PUFAs[237]. Bee (2000) demonstrated that the PUFA (*i.e.*, CLA) fed to female pigs during lactation actually increased the rate of growth of piglets (see 6.3.2, page 28). In rats, Chin *et al.* (1994) and Poulos *et al.* (2001) reported that CLA administered to dams during pregnancy and/or lactation does not elicit an adverse effect to pups, but rather, enhances pup growth (see 6.3.1, page 26). Although the beneficial effects of CLA to infants

[235] The lack of an effect on arachidonic and docosohexanoic acid by CLA is important because these fatty acids play important role in neonatal development.

[236] Although this hypothesis was not tested experimentally, Aneja and Murthi (1990) determined that CLA content of Indian ghee is up to 3.0% of total fat, which is more than six times higher than normal milk.

[237] CLA belongs to a class of fatty acids known as polyunsaturated fatty acids (PUFA).

have not been reported in the literature, several studies have investigated the beneficial effects of PUFAs in infants. Helland *et al.*, (2003) demonstrated that, "*Maternal intake of very-long-chain n3 PUFAs during pregnancy and lactation may be favorable for later mental development of children.*"[238] Importantly, Helland *et al.*, (2001), concluded that long-chain *n*3 PUFAs did not result in any "*harmful*" effect when consumed during pregnancy and lactation. These data do not support an untward effect in humans or experimental animals by CLA or *n*3-PUFAs, but rather, possible beneficial effects to the infant.

In summary, administration of 1.5 g of CLA per day to lactating women in the study by Masters *et al.* (2002) does not appear to have increased the CLA content of breast milk sufficiently high to be considered above biological variation. Experimental evidence in animals does not support that infantile exposure to CLA from breast milk will result in an untoward effect (because long-chain polyunsaturated fatty acids are unaffected in rats and cows), but rather, studies suggest a possible beneficial effect on growth. Variations in the fat content of breast milk is unlikely to result in reduced fat intake in infants, because infants adapt to breast milk content by self-modulating milk intake (Tyson *et al.*, 1992). There is no experimental evidence suggesting that dietary intake of CLA, or other long-chain *n*3 PUFAs, by lactating women will result in an adverse effect to infants. Further, a search of the literature failed to identify a report in which a basis for an untoward effect(s) to infants reared on breast milk low in fat could be based.

8.5. High-Dose, short- to long-term human studies

SECTION OVERVIEW
Animal Studies
6.1 – 6.7 Toxicity – *NOAEL 2.4/2.7 g/kg/d*
Other Biological Studies
7.1 Hepatic lipids – *no toxicological concern*
7.2 Insulin – *no toxicological concern*
7.3 Aortic fat deposition - *equivocal*
7.4 Peroxisomes – *species specific*
7.5 Milk fat – *no toxicological concern*
Human Studies
8.1 Safety – *no adverse effect (AE) 0.1 g/kg/d*
8.2 Insulin – *no toxicological concern*
8.3 Isoprostanes - *equivocal*
8.4 Milk fat - *equivocal*
8.5 Higher dose non-safety
8.6 Lower dose non-safety

In addition to the safety studies by Blankson *et al.* (2000) and Kreider *et al.* (2002), the effect of high doses (*i.e.*, exceeding 6 grams per day) of CLA on non-safety biological parameters has been investigated in athletes. (TABLE 14)

Lowery *et al.*, (1998)[239] supplemented the diet of male bodybuilders with 7.2 grams CLA[240] *per* day[241]

[238] This finding was not supported in earlier studies by Helland *et al.*, (2001) and Auestad *et al.*, (2001).
[239] This article was published in abstract form, thus, information not included in the text above was due to its absence in the original report.
[240] Identity information (*i.e.*, isomers) of the CLA was not reported.

(*n*=12) for six weeks and determined whether muscle development was enhanced. Increases in muscle size and strength were greater in subjects whose diet was supplemented with CLA. Subcutaneous fat, total body fat and body water distribution were unaffected. The authors reported that "*A subset of subjects revealed NS differences in tympanic temperature, or in serum glucose, lipids, BUN:creatinine ratio, LDH, SGOT and SGPT enzymes.*"

Von Loeffelholz *et al.*, (1999) also administered CLA[242] (7 grams *per* day[243]; *n*=7) for 24 weeks (*i.e.*, 6 months) to bodybuilders (males and females) and determined the effect on body composition, strength, blood parameters and energy intake. Body weights, total body water content, fat mass, blood cholesterol, HDL cholesterol, triacylglycerol and energy intake were unaffected by CLA treatment compared to control group[244]. Increases in upper and lower body strength were significantly greater in the CLA group than in the placebo group. Blood chemistry and liver enzyme function were not reported.

Ferreira *et al.*, (1997)[245] administered CLA (6 g/day; *n*=12) to male "*resistance-trained*" athletes for 4 weeks. Body composition, strength, BUN and creatinin were unaffected. Other safety parameters were not measured. Additional information regarding this study is not available. (Table 17)

In summary, CLA administered to athletes at doses up to 7.2 g/day for up to 6 months did not result in any overt adverse effect.

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[241] 7.2 g/day was equivalent to 0.09 g/kg body weight/day (average body weight of athlete was 78 kg).
[242] CLA administered contained 54% CLA, specific isomers were not reported.
[243] 7 g/day was equivalent to 0.084 g/kg body weight/day (average body weight of athlete was 83 kg).
[244] Control group received 7 grams *per* day of sunflower oil.
[245] This study was described in the review by Berven *et al.* (2002).

8.6. Low-Dose, short-term human studies

Previous sections described high dose/long term studies (section 8.1.1). Several additional human studies were selected for critical evaluation, because CLA was administered at a level of 3.6 grams *per* day, for eight weeks or longer. (Table 17)

SECTION OVERVIEW

Animal Studies
6.1 – 6.7 Toxicity – *NOAEL 2.4/2.7 g/kg/d*
Other biological studies
7.1 Hepatic lipids – *no toxicological concern*
7.2 Insulin – *no toxicological concern*
7.3 Aortic fat deposition - *equivocal*
7.4 Peroxisomes – *species specific*
7.5 Milk fat – *no toxicological concern*
Human Studies
8.1 Safety – *no adverse effect (AE) 0.1 g/kg/d*
8.2 Insulin – *no toxicological concern*
8.3 Isoprostanes - *equivocal*
8.4 Milk fat - *equivocal*
8.5 Higher dose non-safety–*no AE 0.09 g/kg/d*
8.6 Lower dose non-safety

8.6.1. Critical evaluation of low-dose, short-term human studies

Noone *et al.* (2002) (see Table 17) performed a double-blind, placebo-controlled study in which two different mixtures of CLA[246] (3 g/day)[247] were ingested daily by healthy[248] male (n=16) and female (n=17) subjects for eight weeks. A single placebo group (7 males and 11 females) that ingested linoleic acid (3 g/day) was also included. Blood samples were obtained prior to starting treatment (week 0), as well as after completing treatment (week 8)[249]. Plasma was analyzed for cholesterol, triacylglycerol, non-esterified fatty acids (NEFA), glucose and insulin. Lipoprotein lipids[250] and fatty acid composition of total plasma lipids[251] were also analyzed.

Supplementation of the diet with 50:50 CLA (3 g/day) for eight weeks reduced[252] plasma triacylglycerol levels 21% compared to baseline; however, plasma triacylglycerol in this group was not significantly different from the placebo group (Noone *et al.*, 2002). Plasma cholesterol, non-esterified fatty acids (NEFA), glucose and insulin were unaffected by either CLA. VLDL-cholesterol (one of the apoproteins quantiated) was reduced 32% in subjects supplemented with the 80:20 CLA (3 g/day); however, VLDL-cholesterol in this group was not significantly different from the placebo group. The other lipoprotein parameters measured

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[246] Two CLA preparations were administered. First, a 50:50 CLA mixture containing *c*9,*t*11-CLA (31%); *t*10,*c*12-CLA (31%) and *t*10,*t*12-CLA (2.4%). A second 80:20 CLA mixture was administered that contained *c*9,*t*11-CLA (44.9%); *t*10,*c*12-CLA (11%) and *t*10,*t*12-CLA (1.1%). Other CLA isoforms were not mentioned.

[247] 3 g/day was equivalent to 0.044 g/kg body weight/day (average body weight for males and females was 68.53 kg).

[248] Volunteers were non-smoking adults (mean age of 31.6 years) with a mean body weight of 68.53 kg and a mean body mass index (BMI) of 23.33 kg/m^2.

[249] Subjects fasted overnight and abstained from drinking alcohol.

[250] Lipoprotein lipids analyzed included VLDL-cholesterol, VLDL-TAG, LDL-cholesterol, HDL-cholesterol, HDL-ApoA-I, HDL_3-cholesterol, HDL_3-ApoA-I, HDL_2-cholesterol, HDL_2-ApoA-I.

[251] Fatty acids analyzed included 16:0, 16:1, 18:0, 18:1, 18:2 (*n*-6), 18:3 (*n*-6), 18:3 (*n*-3), c9,t11-CLA, 20:2, 20:3 (*n*-6), 20:4 (*n*-6), 20:5 (*n*-3), 22:4 (*n*-6), 22:6 (*n*-3).

[252] Use of relative terms (*e.g.*, increase, decrease, reduce) indicates statistical significance from either control or baseline throughout this document.

were unaffected by either CLA treatment. Incorporation of *c*9,*t*11-CLA into plasma lipids was increased 87% and 90% in subjects supplemented with 50:50 CLA and 80:20 CLA, respectively. In subjects treated with 50:50 CLA, incorporation of linolenic acid (18:3 *n*-3) was reduced 32%. In subjects treated with 80:20 CLA, incorporation of eicosa-5Z,8Z,11Z,14Z,17Z-pentaenoic acid into plasma lipids was reduced 24%. Supplementation with CLA had no other effects on fatty acid incorporation into plasma lipids in 50:50-CLA, 80:20-CLA or placebo treatment groups. (Table 17)

CLA supplementation had no effect on body weight. Regarding the effect of CLA on plasma insulin, the investigators reported, *"Our present study demonstrates that in healthy subjects CLA supplementation has neither pro- nor anti-diabetic effects."* (Noone *et al.*, 2002). The investigators did not report the occurrence of any untoward effect(s) due to CLA supplementation.

This study by Noone *et al.* (2002) was limited by (1) the small number of subjects that participated, (2) the short period of supplementation and, (3) the standard battery of clinical chemistry, serum enzyme parameters and occurrence of adverse events were not reported. Nevertheless, the findings that are presented do not indicate that CLA, at 3 grams *per* day, produces any untoward effect in humans.

Kamphuis *et al.* (2003a) (see Table 17) performed a randomized, double-blind, placebo-controlled study in which CLA[253] (1.8 or 3.6 g/day)[254] was ingested daily by overweight[255] male (*n*=26) and female (*n*=28) volunteers (*a.k.a.* subjects) for thirteen (13) weeks. Three weeks prior to initiating treatment, subjects were fed a very-low-calorie diet (VLCD) to produce a reduction in body weight prior to commencing CLA treatment. Two placebo groups were also included that ingested 1.8 or 3 g/day oleic acid. Body weight, appetite[256] and energy intake[257] were recorded prior to starting treatment (week 0), during treatment (weeks 3 and 8) and after treatment (week 13).

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[253] CLA administered was Tonalin™ TG 75 (*i.e.*, 75% CLA 50:50). Specific CLA isomers were not reported.
[254] 1.8 and 3.6 g/day were equivalent to 0.021 and 0.042 g/kg body weight/day, respectively.
[255] Subjects had a mean body weight of 85.1 kg, mean BMI of 27.8 kg/m^2 and mean body fat of 31.3%.
[256] Appetite was assessed using a questionnaire.
[257] Energy intake was measured at breakfast using a Universal Eating Monitor (Westerterp-Plantenga, 2000).

An increase in the occurrence of adverse events did not result from CLA (3.6 g/day) supplementation. Feelings of satiety and fullness were increased, while hunger was decreased in subjects supplemented with CLA (3.6 g/day) as compared to the placebo group. Energy intake and body weight regain were unaffected by CLA supplementation. Other safety parameters (clinical chemistry and serum liver enzymes) were not reported by Kamphuis *et al.* (2003a). Because CLA had no effect on the occurrence of adverse events, CLA, even at the high dose of 3.6 g/day, was well tolerated throughout the 13-week supplementation period.

Kamphuis *et al.* (2003b) (see Table 17) published a second report in which the effects of CLA supplementation on body weight regain, body composition and resting metabolic rate in overweight subjects[258] were investigated (see page 93 for protocol). Body weight, body mass index, body fat (%), absolute fat mass, fat-free mass, resting metabolic rate and, plasma glucose, insulin, triglyceride, free fatty acid, glycerol and β-hydroxybutyrate, were measured prior to starting treatment (week 0) and after treatment (week 13).

Body weight regain was unaffected by CLA supplementation. After 13 weeks of supplementation with CLA (1.8 and 3.6 g/day), body fat mass was reduced when expressed as a percentage, but was unaffected when expressed as absolute fat mass. Fat-free mass was increased by CLA (1.8 or 3.6 g/day) supplementation. Although resting metabolic rate was increased in CLA supplemented subjects, it was determined to be due to the increase in fat-free mass rather than to CLA. Plasma glucose, insulin, triglyceride, free fatty acid, glycerol and β-hydroxybutyrate were unaffected by CLA after 13 weeks of supplementation. Other safety parameters (clinical chemistry and serum liver enzymes) were not reported by Kamphuis *et al.* (2003b). These data indicated that 1.8 or 3.6 g/day CLA for 13 weeks did not produce untoward effects in overweight humans.

[258] Subjects had a mean body weight of 85.1 kg, mean BMI of 27.8 kg/m^2 and mean body fat of 31.3%.

8.7. Summary of observations in humans

SECTION OVERVIEW
Animal Studies
6.1 – 6.7 Toxicity – *NOAEL 2.4/2.7 g/kg/d*
Other Biological Studies
7.1 Hepatic lipids – *no toxicological concern*
7.2 Insulin – *no toxicological concern*
7.3 Aortic fat deposition - *equivocal*
7.4 Peroxisomes – *species specific*
7.5 Milk fat – *no toxicological concern*
Human Studies
8.1 Safety – *no adverse effect (AE) 0.1 g/kg/d*
8.2 Insulin – *no toxicological concern*
8.3 Isoprostanes - *equivocal*
8.4 Milk fat - *equivocal*
8.5 Higher dose non-safety–*no AE 0.09 g/kg/d*
8.6 Lower dose non-safety-*no AE 0.04 g/kg/d*

No observable adverse effect has been reported in normal healthy humans administered CLA (6.8 g/day) for 12 weeks (3 months) (see Table 17). Recent studies indicate that supplementation of the diet with 3.4 g/day CLA for 104 weeks (24 months) does not produce any untoward effect in normal healthy subjects. Although adverse events reported were gastrointestinal-related, the incidence of adverse events was not treatment related. The moderate increases in plasma insulin and endogenous isoprostanes have been reported in humans treated with CLA, yet, no pathology or functional effect has been demonstrated. Although CLA is reported to reduce breast milk fat content in humans, results from experimental animal studies in rats and cows indicate that this result is will not likely result in an adverse effect because long-chain polyunsaturated fatty acids are unaffected by CLA treatment.

9. Safety evaluation-humans

CLA is a mixture of isomeric fatty acid substances in which the predominant substances are the *c*9,*t*11- and *t*10,*c*12-CLA isomers. CLA can be manufactured as free fatty acids or conjugated with glycerol. Although glycerol is an innocuous substance, it might influence the actions of CLAs esterified to it. Thus, the present safety assessment also evaluated the potential effects of either free fatty acid CLA or CLA esterified with glycerol.

Experimental studies indicate that CLA is well absorbed by the gastrointestinal tract, distributed in adipose tissue, as well as the lipid compartment(s) of liver, kidney and other organs. CLA is rapidly converted into CO_2 and eliminated from the body in expired air. A smaller amount of CLA appears to be biotransformed into other unidentified water-soluble component(s) that is excreted in the urine. The extensive conversion of CLA to CO_2 indicates that the isomer constituents of CLA are substrates for β-oxidation *via* the Kreb's cycle. CLA appears to be metabolized *via* a biochemical pathway that is generally common to fatty acids. It is assumed that physiological responses are similar between experimental animals and humans.

There is extensive literature on the use of CLA as a dietary supplement. It is reported to be without adverse effect in dogs, guinea pigs, pigs, rabbits and rats at doses up to 8.4 g/kg.

O'Hagan and Menzel (2003) used a "*conservative approach*" to determine the NOAEL for CLA to be about 2.6 g/kg for rats. CLA does not produce a teratogenic effects or adverse developmental effect in rats. CLA does not produce teratogenic effect or developmental adverse effects in rats; CLA is non-mutagenic and it is not a dermal irritant. High concentrations of CLA have been shown to affect hepatocyte viability and growth *in vitro*. There is also evidence for species specificity regarding CLA's effect on hepatic lipid accumulation, increase in insulin concentration and aortic fat deposition.

The biological effect of CLA in humans has been reported in numerous studies. Supplementation of the diet with high doses of CLA (7-7.2 g/day for up to 24 weeks) in athletes did not result in any overt sign of toxicity. Similarly, consumption of 6.8 g/day CLA for 12 weeks by normal healthy subjects did not result in any sign of an adverse effect assessed by traditional clinical safety parameters. Administration of CLA (3.4 g/day) for up to 104 weeks, likewise, does not result in any adverse effect.

Although reduced breast milk fat has been demonstrated in mice, rats, cows and women, there is no experimental animal or human evidence to indicate that this effect adversely affects the neonate. In contrast, experimental animal evidence indicates a possible growth enhancement effect of CLA to neonates.

Data from human clinical studies indicate that consumption of 3-7.2 g/day (*i.e.*, 0.05-0.1 g/kg body weight/day) CLA does not produce any adverse effect in humans. Based on this critical evaluation of the available scientific and biomedical evidence, the proposed use of Tonalin® TG 80 in selected fruit juices, meal replacement products, yogurts and chocolate is safe for human consumption by the oral route.

10. Conclusion

The Expert Panel concludes from its critical evaluation of the relevant scientific literature and information summarized above that the use of CLA as Tonalin® TG 80 in fruit juices, meal replacement products, yogurts and chocolate, as the concentrations specified[259], resulting in a 90th percentile consumption of 4.93g/day (3.94g CLA) and the estimated safe total daily consumption is 8.75g Tonalin® TG 80 (7.0g CLA), meeting the specifications described herein, produced under current Good Manufacturing Practice (21CFR110)[260] is generally recognized as safe (GRAS) by scientific procedures.

Signatures:

(b) (6)

04 June 2004

Joseph Borzelleca, Ph.D., F.A.T.S. Date
Consultant

(b) (6)

5/26/04

John Thomas, Ph.D., F.A.T.S. Date
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5/27/04

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Consultant

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12. APPENDIX A. FOODS SELECTED FOR CONSUMPTION ANALYSIS

FOODCODE†	DESCRIPTION	Serving Size (g)‡	CONCENTRATION (mg/serving)‡	CLA (mg/g)
11410000	YOGURT NS AS TO TYPE OF MILK/FLAVOR	225	2100	9.33
11411010	YOGURT PLAIN NS AS TO TYPE OF MILK	225	2100	9.33
11411100	YOGURT PLAIN WHOLE MILK	225	2100	9.33
11411200	YOGURT PLAIN LOWFAT MILK	225	2100	9.33
11411300	YOGURT PLAIN NONFAT MILK	225	2100	9.33
11425000	YOGURT CHOCOLATE NS AS TO TYPE OF MILK	225	2100	9.33
11427000	YOGURT CHOCOLATE NONFAT MILK	225	2100	9.33
11431000	YOGURT FRUIT VARIETY WHOLE MILK	225	2100	9.33
11432000	YOGURT FRUIT VARIETY LOWFAT MILK	225	2100	9.33
11433500	YOGURT FRUITED NONFAT MILK LOW CAL SWEETENER	225	2100	9.33
11511200	MILK CHOCOLATE REDUCED FAT MILK BASED	240	2100	8.75
11511300	MILK CHOCOLATE SKIM MILK BASED	240	2100	8.75
11551050	MILK FRUIT DRINK (INCL LICUADO)	240	2100	8.75
11552200	MILK-BASED FRUIT DRINK (INCL ORANGE JULIUS)	240	2100	8.75
11611000	INSTANT BREAKFAST FLUID CANNED	240	2100	8.75
11612000	INSTANT BREAKFAST POWDER MILK ADDED	240	2100	8.75
11613000	INSTANT BFAST PWDR SWT W/ LO CAL SWT MILK ADDED	240	2100	8.75
11623000	MEAL SUPPLEMENT / REPLACEMENT PREPARED RTD	240	2100	8.75
11641000	MEAL REPLACEMENT MILK BASED HIGH PROTEIN LIQUID	240	2100	8.75
11830800	INSTANT BREAKFAST POWDER NOT RECONSTITUTED	240	2100	8.75
11830810	INSTANT BFAST PWDR SWT W/ LO CAL SWT NOT RECONSTUT	240	2100	8.75
11830900	PROTEIN SUPPLEMENT MILK BASED DRY POWDER	240	2100	8.75
11830940	MEAL REPLACEMENT PROTEIN MILK BASED FRUIT JUICE MIX	240	2100	8.75
11830970	MEAL REPLACEMENT PROTEIN TYPE MILK-BASE POWDER	240	2100	8.75
11832000	MEAL REPLACEMENT MILK-&SOY-BASE POWDER NOT RECONST	240	2100	8.75
11835100	MEAL REPLACEMENT POSITRIM DRINK MIX DRY POWDER	240	2100	8.75
12210200	CREAM SUBSTITUTE LIQUID (INCLUDE COFFEE WHITNER)	15	500	33.33
12210400	CREAM SUBSTITUTE POWDERED	2	500	66.67
41435110	HIGH PROTEIN BAR CANDY-LIKE SOY & MILK BASE	40	2100	52.50
41440010	MEAL REPLACEMENT/SUPPLEMENT LIQUID HI PROTEIN	240	2100	8.75
41440100	MEAL REPLACEMENT LIQUID SOY-BASE (ISOCAL OSMOLITE)	240	2100	8.75
53260030	COOKIE DIETETIC CHOCOLATE CHIP	30	2100	70.00
53540000	BREAKFAST BAR NFS	40	2100	52.50
53541100	BREAKFAST BAR DIET MEAL TYPE	40	2100	52.50
53541200	MEAL REPLACEMENT BAR (INCL SLIM FAST BAR)	40	2100	52.50
61201000	GRAPEFRUIT JUICE NFS	240	2100	8.75
61201020	GRAPEFRUIT JUICE UNSWEETENED NS AS TO FORM	240	2100	8.75
61201220	GRAPEFRUIT JUICE CANNED BOTTLED CARTON UNSWEET	240	2100	8.75
61201230	GRAPEFRUIT JUICE CANNED BOTTLED CARTON W/ SUGAR	240	2100	8.75
61201240	GRAPEFRUIT JUICE CANNED/BOTTLE/CARTON W/ LOW CAL SWEETENER	240	2100	8.75
61210000	ORANGE JUICE NFS	240	2100	8.75
61210220	ORANGE JUICE CANNED/BOTTLED/CARTON UNSWEETENED	240	2100	8.75

61210230	ORANGE JUICE CANNED/BOTTLED/CARTON W/ SUGAR	240	2100	8.75
61210250	ORANGE JUICE W/ CALCIUM CAN/BOTTLE/CARTON UNSWEETENED	240	2100	8.75
61216000	GRAPEFRUIT & ORANGE JUICE NFS	240	2100	8.75
61216220	GRAPEFRUIT & ORANGE JUICE CANNED UNSWEETENED	240	2100	8.75
64100100	FRUIT JUICE NFS (INCLUDE MIXED FRUIT JUICES)	240	2100	8.75
64100110	FRUIT JUICE BLEND 100% JUICE W/ VITAMIN C	240	2100	8.75
91705010	CHOCOLATE MILK PLAIN	40	500	12.50
91705020	CHOCOLATE MILK W/ CEREAL (INCLUDE KRACKEL BAR)	40	500	12.50
91705040	CHOCOLATE MILK W/ NUTS NOT ALMONDS OR PEANUTS	40	500	12.50
91705050	CHOCOLATE MILK W/ FRUITS & NUTS (INCLUDE CHUNKY)	40	500	12.50
91705060	CHOCOLATE MILK W/ ALMONDS	40	500	12.50
91705070	CHOCOLATE MILK W/ PEANUTS (INCLUDE MR GOODBAR)	40	500	12.50
91705200	CHOCOLATE SEMI-SWEET	40	500	12.50
91705300	CHOCOLATE CANDY SWEET OR DARK	40	500	12.50
91705400	CHOCOLATE CANDY WHITE	40	500	12.50
91705410	CHOCOLATE CANDY WHITE W/ ALMONDS	40	500	12.50
91705420	CHOCOLATE WHITE W/ CEREAL CANDY	40	500	12.50
91770030	DIETETIC OR LOW CALORIE CANDY CHOCOLATE-COVERED	40	500	12.50
92511190	ORANGE JUICE DRINK	240	2100	8.75
92511250	CITRUS FRUIT JUICE DRINK (INCL 5-ALIVE)	240	2100	8.75
92520410	FRUIT DRINK LOW CALORIE	240	2100	8.75
92530410	CITRUS DRINK W/ VITAMIN C ADDED	240	2100	8.75
92530810	GRAPEFRUIT JUICE DRINK W/ VITAMIN C ADDED	240	2100	8.75
92531010	ORANGE DRINK & ORANGEADE W/ VITAMIN C ADDED	240	2100	8.75
92531020	ORANGE BREAKFAST DRINK FROM FROZEN CONCENTRATE	240	2100	8.75
92531030	ORANGE BREAKFAST DRINK	240	2100	8.75
92550300	GRAPEFRUIT JUICE DRINK LOW CALORIE W/ VITAMIN C	240	2100	8.75
92550610	FRUIT-FLAVORED DRINK LOW CAL W/ VITAMIN C ADDED	240	2100	8.75
92551600	CITRUS JUICE DRINK LOW CALORIE	240	2100	8.75
92551700	JUICE DRINK LOW CALORIE	240	2100	8.75
92552050	ORANGE BREAKFAST DRINK LOW CALORIE	240	2100	8.75
92582110	ORANGE BREAKFAST DRINK CALCIUM FORTIFIED	240	2100	8.75

†Continuing Survey of Food Intake (CSFII) 1994-1996 database
‡Title 21 of the Code of Federal Regulations (CFR) section 101.12, 2003 Edition
*Provided by Cognis Corporation

13. APPENDIX B. RESULTS OF FIVE BATCH ANALYSES

BATCH		SR 63: GR31348901, distilled	SR 64: GR31368901, distilled	SR 65: GR31408901, distilled	SR 66: GR31468901, distilled	SR 67: GR31718901, distilled
Fatty acid profile, GC (100 m column)						
C16:0	rel.-area %	1.5	1.5	1.6	1.5	1.3
C18:0	rel.-area %	2.9	2.9	2.9	2.8	2.9
C18:1, c9	rel.-area %	13.2	13.0	13.1	13.0	12.6
C18:2, c9c12	rel.-area %	0.4	0.4	0.3	0.3	0.2
CLA, total	rel.-area %	78.5	79.0	79.0	79.3	80.0
CLA c9, t11	rel.-area %	38.4	38.6	38.9	38.9	39.2
CLA t10,c12	rel.-area %	37.9	38.4	38.1	38.4	39.0
CLA c,c isomers**	rel.-area %	1.1	1.0	1.0	1.0	0.9
CLA t,t isomers	rel.-area %	1.0	0.9	0.9	0.9	0.9
CLA t8, c10	rel.-area %	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
CLA c11, t13	rel.-area %	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
TG-content	rel.-area %	92.1	92.8	84.6	83.5	89.1
DG-content	rel.-area %	6.5	5.5	13.3	14.9	8.8
MG-content	rel.-area %	0.0	0.0	0.1	0.1	0.0
FFA	rel.-area %	0.5	0.7	1.0	0.7	0.7
glycerol	rel.-area %	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Analysis						
AV	mg KOH / g	0.40	0.66	1.21	0.85	0.62
SV	mg KOH / g	188.8	190.3	191.3	190.5	189.3
POV	meq / kg	0.0	0.1	0.0	0.0	0.0
IV	g I2 / 100g	163	161	162	163	166
Color (Gardner)		0.0	0.0	0.0	0.0	0.0
Unsaponifiable matter	w-%	0.4	0.3	0.2	0.3	0.6
Heavy metal as Pb	mg/kg	< 1	< 1	< 1	< 1	< 1
PAH						
Light	µg/kg	< 0.5*	< 0.5*	< 0.5*	< 0.5*	< 0.5*
Heavy	µg/kg	2.1	2.4	< 0.5*	< 0.5*	2.1
BaP	µg/kg	0.7	0.8	< 0.5*	< 0.5*	0.7
Monitoring						
Pesticides	mg/kg	ND	ND	ND	ND	ND
Pb	mg/kg	< 0.1*	< 0.1*	< 0.1*	< 0.1*	< 0.1*
As	mg/kg	<0.1*	<0.1*	<0.1*	<0.1*	<0.1*
Cd	mg/kg	<0.01*	<0.01*	<0.01*	<0.01*	<0.01*
Hg	mg/kg	<0.01*	<0.01*	<0.01*	<0.01*	<0.01*
Sn	mg/kg	<0.2*	<0.2*	<0.2*	<0.2*	<0.2*
Residual ethanol	µg/g	< 50*	< 50*	< 50*	< 50*	< 50*
Dioxine, furane	ng WHO-TE/kg	0.140	0.139	0.134	0.137	0.142
Aflatoxine						
B1	µg/kg	< 0.1*	< 0.1*	< 0.1*	< 0.1*	< 0.1*
B2	µg/kg	< 0.1*	< 0.1*	< 0.1*	< 0.1*	< 0.1*
G1	µg/kg	< 0.1*	< 0.1*	< 0.1*	< 0.1*	< 0.1*
G2	µg/kg	< 0.1*	< 0.1*	< 0.1*	< 0.1*	< 0.1*
M1	µg/kg	< 0.01*	< 0.01*	< 0.01*	< 0.01*	< 0.01*
Total aerobic microbial count	cfu/g	< 100	< 100	< 100	< 100	< 100
Molds, yeasts	cfu/g	< 100	< 100	< 100	< 100	< 100

BATCH		SR 63: GR31348901, distilled	SR 64: GR31368901, distilled	SR 65: GR31408901, distilled	SR 66: GR31468901, distilled	SR 67: GR31718901, distilled
Coliforms	cfu/g	neg	neg	neg	neg	neg
E. Coli	cfu/g	neg	neg	neg	neg	neg
Staphylcoccus. aureus	cfu/10g	neg	neg	neg	neg	neg
Salmonella	cfu/25g	neg	neg	neg	neg	neg
Aerobic spore formers	cfu/g	< 100	< 100	< 100	< 100	< 100
Additional Analytic						
Color (Lovibond) 1´´						
Yellow		0.3	0.3	0.2	0.2	0.5
Red		0.1	0.1	0.1	0.1	0.1
Qater	%	0.02	0.01	0.01	0.01	0.01
n_D^{20}		1.4877	1.4877	1.4876	1.4876	1.4877
Density (20°C)	g/cm³	0.9222	0.9220	0.9222	0.9225	0.9222
vVscosity (20°C)	cps	143	142	142	143	144
Ash (600°C)	%	0.00	0.00	0.00	0.00	0.01

* value correspond to detection limit.
**values too high, because of overlapping of cc-isomers and t10,c12
c,c-isomers; t,t-isomers:
values of 1,1 or 1,2 % are also realistic. My proposal for GRAS: < 1,5%.
Data was provided by Cognis Corporation Nutrition and Health.

14. APPENDIX C. DETAILED CONSUMPTION ANALYSIS REPORT

CONSUMPTION ANALYSES REPORT

Source: USDA Continuing Survey of Food Intakes by Individuals (CSFII) 1994-1996, CD-ROM

Analysis Parameters

Description of Food(s)	All Foods
Years	1994-1996
Concentration (mg/g)	8.75-70

Per Capita Consumption Estimate of Ingredient from Selected Foods+

	Eaters Only+	
	(g/day)	(g/kg/day)*
Mean	2.72	0.045
90th Percentile	4.93	0. 082

+Estimate is based on individuals in the U.S. that consume this food, commonly referred to as "*eaters only*" data.

++Based on 1996 U. S. population of 265,462,901 (www.census.gov/cgi-bin/pc/idbrank.pl).

*Assuming average body weight of 60 kilograms(kg)

Total Individuals That Consume All Foods	327998880.00
Total Amount of Ingredient Consumed (g)	8.93×10^8

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15. APPENDIX D. TARGET FOODS SUMMARY

FOODCODE	DESCRIPTION	SERVING SIZE (g)	NEW CONCENTRATION LEVEL (mg/serving)	CONCENTRATION (mg/g)	INGREDIENT CONSUMED (mg)	PERCENT OF TOTAL
11410000	YOGURT NS AS TO TYPE OF MILK/FLAVOR	225	2100	9.33	426346303.88	0.0477%
11411010	YOGURT PLAIN NS AS TO TYPE OF MILK	225	2100	9.33	559584978.12	0.0627%
11411100	YOGURT PLAIN WHOLE MILK	225	2100	9.33	1884847624.8	0.2111%
11411200	YOGURT PLAIN LOWFAT MILK	225	2100	9.33	4893845060.81333	0.5481%
11411300	YOGURT PLAIN NONFAT MILK	225	2100	9.33	4547635504.44	0.5093%
11425000	YOGURT CHOCOLATE NS AS TO TYPE OF MILK	225	2100	9.33	146666800	0.0164%
11427000	YOGURT CHOCOLATE NONFAT MILK	225	2100	9.33	617574591.386667	0.0692%
11431000	YOGURT FRUIT VARIETY WHOLE MILK	225	2100	9.33	2912860356.74667	0.3262%
11432000	YOGURT FRUIT VARIETY LOWFAT MILK	225	2100	9.33	24586507647.3334	2.7535%
11433500	YOGURT FRUITED NONFAT MILK LOW CAL SWEETENER	225	2100	9.33	16926431831.4	1.8956%
11511200	MILK CHOCOLATE REDUCED FAT MILK BASED	240	2100	8.75	51959353338.4625	5.8190%
11511300	MILK CHOCOLATE SKIM MILK BASED	240	2100	8.75	2055595864	0.2302%
11551050	MILK FRUIT DRINK (INCL LICUADO)	240	2100	8.75	160460011.25	0.0180%
11552200	MILK-BASED FRUIT DRINK (INCL ORANGE JULIUS)	240	2100	8.75	2316228521.25	0.2594%
11611000	INSTANT BREAKFAST FLUID CANNED	240	2100	8.75	293520937.5	0.0329%
11612000	INSTANT BREAKFAST POWDER MILK ADDED	240	2100	8.75	1920178361.325	0.2150%
11613000	INSTANT BFAST PWDR SWT W/ LO CAL SWT MILK ADDED	240	2100	8.75	52382881.25	0.0059%
11623000	MEAL SUPPLEMENT / REPLACEMENT PREPARED RTD	240	2100	8.75	9743015812.8625	1.0911%
11641000	MEAL REPLACEMENT MILK BASED HIGH PROTEIN LIQUID	240	2100	8.75	311454080	0.0349%
11830800	INSTANT BREAKFAST POWDER NOT RECONSTITUTED	240	2100	8.75	1072452586.625	0.1201%
11830810	INSTANT BFAST PWDR SWT W/ LO CAL SWT NOT RECONSTUT	240	2100	8.75	45451472.5	0.0051%
11830900	PROTEIN SUPPLEMENT MILK BASED DRY POWDER	240	2100	8.75	132031132.625	0.0148%
11830940	MEAL REPLACEMENT PROTEIN MILK BASED FRUIT JUICE MIX	240	2100	8.75	3226965	0.0004%
11830970	MEAL REPLACEMENT PROTEIN TYPE MILK-BASE POWDER	240	2100	8.75	824948046.5625	0.0924%
11832000	MEAL REPLACEMENT MILK-&SOY-BASE POWDER NOT RECONST	240	2100	8.75	140982568.125	0.0158%
11835100	MEAL REPLACEMENT POSITRIM DRINK MIX DRY POWDER	240	2100	8.75	125633261.25	0.0141%

FOODCODE	DESCRIPTION	SERVING SIZE (g)	NEW CONCENTRATION LEVEL (mg/serving)	CONCENTRATION (mg/g)	INGREDIENT CONSUMED (mg)	PERCENT OF TOTAL
12210200	CREAM SUBSTITUTE LIQUID (INCLUDE COFFEE WHITNER)	15	500	33.33	22340007744.6667	2.5019%
12210400	CREAM SUBSTITUTE POWDERED	2	500	66.67	30890102205.2222	3.4594%
41435110	HIGH PROTEIN BAR CANDY-LIKE SOY & MILK BASE	40	2100	52.50	147482577.375	0.0165%
41440010	MEAL REPLACEMENT/SUPPLEMENT LIQUID HI PROTEIN	240	2100	8.75	4600209915	0.5152%
41440100	MEAL REPLACEMENT LIQUID SOY-BASE (ISOCAL OSMOLITE)	240	2100	8.75	1317159173.75	0.1475%
53260030	COOKIE DIETETIC CHOCOLATE CHIP	30	2100	70.00	43747200	0.0049%
53540000	BREAKFAST BAR NFS	40	2100	52.50	287391830.775	0.0322%
53541100	BREAKFAST BAR DIET MEAL TYPE	40	2100	52.50	344395209.375	0.0386%
53541200	MEAL REPLACEMENT BAR (INCL SLIM FAST BAR)	40	2100	52.50	3354448939.275	0.3757%
61201000	GRAPEFRUIT JUICE NFS	240	2100	8.75	586642138.075	0.0657%
61201020	GRAPEFRUIT JUICE UNSWEETENED NS AS TO FORM	240	2100	8.75	1283660723.625	0.1438%
61201220	GRAPEFRUIT JUICE CANNED BOTTLED CARTON UNSWEET	240	2100	8.75	21825901042.4	2.4443%
61201230	GRAPEFRUIT JUICE CANNED BOTTLED CARTON W/ SUGAR	240	2100	8.75	2071671106.05	0.2320%
61201240	GRAPEFRUIT JUICE CANNED/BOTTLE/CARTON W/ LOW CAL SWEETENER	240	2100	8.75	361705601.25	0.0405%
61210000	ORANGE JUICE NFS	240	2100	8.75	47193144869.675	5.2853%
61210220	ORANGE JUICE CANNED/BOTTLED/CARTON UNSWEETENED	240	2100	8.75	400495688446.787	44.8524%
61210230	ORANGE JUICE CANNED/BOTTLED/CARTON W/ SUGAR	240	2100	8.75	2330070561	0.2609%
61210250	ORANGE JUICE W/ CALCIUM CAN/BOTTLE/CARTON UNSWEETENED	240	2100	8.75	2687971811.875	0.3010%
61216000	GRAPEFRUIT & ORANGE JUICE NFS	240	2100	8.75	71196437.8125	0.0080%
61216220	GRAPEFRUIT & ORANGE JUICE CANNED UNSWEETENED	240	2100	8.75	690741983.75	0.0774%
64100100	FRUIT JUICE NFS (INCLUDE MIXED FRUIT JUICES)	240	2100	8.75	23210783362.2	2.5994%
64100110	FRUIT JUICE BLEND 100% JUICE W/ VITAMIN C	240	2100	8.75	30740987519.8125	3.4427%
91705010	CHOCOLATE MILK PLAIN	40	500	12.50	8239664753	0.9228%
91705020	CHOCOLATE MILK W/ CEREAL (INCLUDE KRACKEL BAR)	40	500	12.50	3163526103.375	0.3543%
91705040	CHOCOLATE MILK W/ NUTS NOT ALMONDS OR PEANUTS	40	500	12.50	1906521756.25	0.2135%
91705050	CHOCOLATE MILK W/	40	500	12.50	415189814.25	0.0465%

FOODCODE	DESCRIPTION	SERVING SIZE (g)	NEW CONCENTRATION LEVEL (mg/serving)	CONCENTRATION (mg/g)	INGREDIENT CONSUMED (mg)	PERCENT OF TOTAL
91705060	FRUITS & NUTS (INCLUDE CHUNKY) CHOCOLATE MILK W/ ALMONDS	40	500	12.50	2825212775.75	0.3164%
91705070	CHOCOLATE MILK W/ PEANUTS (INCLUDE MR GOODBAR)	40	500	12.50	1066558722.5	0.1194%
91705200	CHOCOLATE SEMI-SWEET	40	500	12.50	425817457.75	0.0477%
91705300	CHOCOLATE CANDY SWEET OR DARK	40	500	12.50	118809032.5	0.0133%
91705400	CHOCOLATE CANDY WHITE	40	500	12.50	130845246.25	0.0147%
91705410	CHOCOLATE CANDY WHITE W/ ALMONDS	40	500	12.50	28532208	0.0032%
91705420	CHOCOLATE WHITE W/ CEREAL CANDY	40	500	12.50	221285161	0.0248%
91770030	DIETETIC OR LOW CALORIE CANDY	40	500	12.50	16278825	0.0018%
92511190	CHOCOLATE-COVERED ORANGE JUICE DRINK	240	2100	8.75	9437272863.6375	1.0569%
92511250	CITRUS FRUIT JUICE DRINK (INCL 5-ALIVE)	240	2100	8.75	7453070876.9125	0.8347%
92520410	FRUIT DRINK LOW CALORIE	240	2100	8.75	2060111418.75	0.2307%
92530410	CITRUS DRINK W/ VITAMIN C ADDED	240	2100	8.75	11352224336.25	1.2714%
92530810	GRAPEFRUIT JUICE DRINK W/ VITAMIN C ADDED	240	2100	8.75	5763460590.125	0.6455%
92531010	ORANGE DRINK & ORANGEADE W/ VITAMIN C ADDED	240	2100	8.75	12971870710.575	1.4527%
92531020	ORANGE BREAKFAST DRINK FROM FROZEN CONCENTRATE	240	2100	8.75	1782777908.1875	0.1997%
92531030	ORANGE BREAKFAST DRINK	240	2100	8.75	66658934521.725	7.4653%
92550300	GRAPEFRUIT JUICE DRINK LOW CALORIE W/ VITAMIN C	240	2100	8.75	426135084.375	0.0477%
92550610	FRUIT-FLAVORED DRINK LOW CAL W/ VITAMIN C ADDED	240	2100	8.75	26895609316.275	3.0121%
92551600	CITRUS JUICE DRINK LOW CALORIE	240	2100	8.75	239194200	0.0268%
92551700	JUICE DRINK LOW CALORIE	240	2100	8.75	700035787.5	0.0784%
92552050	ORANGE BREAKFAST DRINK LOW CALORIE	240	2100	8.75	2748281754.75	0.3078%
92582110	ORANGE BREAKFAST DRINK CALCIUM FORTIFIED	240	2100	8.75	338591568	0.0379%

***Foodcodes- 11553000, 11553100, 11551100, 11560100, 11521010, 53260050, 61216230, 61216620 and 91705090 were queried and no data was found.*

SUBMISSION END

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Food and Drug Administration, HFS 255
5100 Paint Branch Parkway
College Park, MD 20740-3835

August 16, 2004

Dear Dr. Harris,

Pursuant to our conversation I would like to withdraw GRAS notification no.153 for conjugated linoleic acid. I look forward to meeting with you in the near future as we discussed.

If there are any questions please do not hesitate to contact me.

Best Regards,

(b)(6)

Heather Nelson Cortes, Ph.D.

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